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        FILE 'BIOSIS, MEDLINE, CAPLUS, WPIDS' ENTERED AT 11:13:43 ON 18 JUL 2003
                  E BUTLIN L/AU
L1            4 S E4-E5
                  E COLEY J/AU
                  E COLEY JOHN/AU
L2           40 S E3-E5
                  E EIDA STEPHEN/AU
L3            5 S E3-E5
                  E GANI MOHAMED/AU
L4            9 S E3-E6
L5           49 S L1-L4
L6           42 DUP REM L5 (7 DUPLICATES REMOVED)
L7            9 S L6 AND ASSAY
L8      1035471 S ASSAY OR IMMUNOASSAY
L9            0 S L8 AND (MENOPAUSE P HORMONE#)
L10        1268 S L8 AND MENOPAUSE
L11         120 S L10 AND FOLLICLE STIMULATING HORMONE
L12         104 DUP REM L11 (16 DUPLICATES REMOVED)
L13           4 S L12 AND ISOFORM#
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Brief Summary Text - BSTX (3):

The present invention relates to an immunoassay for determining the relative concentrations of two different immunogenic <u>analytes</u>. In particular, the invention relates to an assay for determining relative concentrations, in a single sample, of related hormonal metabolites such as pregnanediol-3-glucuronide (P-3-G) and estrone-3- glucuronide (E.sub.1 -3-G). Even more particularly, the invention relates to an immunoassay suitable for testing for constituents in human urine to determine the human fertile period, that is, the period in which viable sperm and a viable ovum may be present simultaneously in the female reproductive tract.

Brief Summary Text - BSTX (5):

L13   ANSWER 1 OF 4   MEDLINE
AN   97002173   MEDLINE
DN   97002173   PubMed ID: 8849573
TI   FSH **isoforms**: bio and immuno-activities in post-menopausal and
      normal menstruating women.
AU   Creus S; Pellizzari E; Cigorraga S B; Campo S
CS   Centro de Investigaciones Endocrinologicas, Hospital General de Ninos R.
      Gutierrez, Buenos Aires, Argentina.
SO   CLINICAL ENDOCRINOLOGY, (1996 Feb) 44 (2) 181-9.
      Journal code: 0346653. ISSN: 0300-0664.
CY   ENGLAND: United Kingdom
DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Priority Journals
EM   199701
ED   Entered STN: 19970128
      Last Updated on STN: 19970128
      Entered Medline: 19970102

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L13   ANSWER 1 OF 4   MEDLINE
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DN   97002173   PubMed ID: 8849573
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AU   Creus S; Pellizzari E; Cigorraga S B; Campo S
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DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Priority Journals
EM   199701
ED   Entered STN: 19970128
      Last Updated on STN: 19970128
      Entered Medline: 19970102
AB   OBJECTIVE: The full expression of gonadotrophin biological activity
      depends on the gonadotrophin carbohydrate component. Our aim was to
study
      serum FSH **isoforms** present in the follicular phase (FPS) and in
      the **menopause** (PMS) since the endocrine status may influence the
      structure of incorporated oligosaccharides. SUBJECTS: Ten healthy
      post-menopausal women (age range 53-68) not receiving any hormonal
      treatment and 10 healthy women (age range 20-28) in the follicular phase
      of their menstrual cycle were studied. MEASUREMENTS: Bio and immuno
      FSH-activities (Sertoli cell aromatase induction **assay** and RIA,
      respectively) were determined in separated **isoforms** after
      concanavalin A chromatography. Isolated **isoforms** were: UB,

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unbound; WB, weakly bound and FB, firmly bound to the lectin. RESULTS:
PMS showed two groups of immuno and bio-active FSH **isoforms**: WB,
bearing biantennary galactosylated type and FB, bearing high mannose or
hybrid type oligosaccharides. Immuno and bio-active FSH were not
detected
in the UB fractions. WB **isoforms** represented 82 +/- 6% of the
total bioactivity recovered in samples analysed individually; their B/I
ratio was 0.85 +/- 0.20. FB **isoforms** were 18 +/- 6%; their B/I
ratio was 3.27 +/- 0.60. Whole serum B/I ratio was 1.20 +/- 0.30.
Similar results were obtained when pooled sera was analysed: WB: 77%,
B/I:
0.82; FB: 23%, B/I: 3.75. Whole serum B/I in pooled samples was 1.10.
FPS showed a different pattern. UB **isoforms,** bearing
triantennary or bisecting oligosaccharides, were 41 +/- 3% of the total
bioactivity recovered in samples analysed individually. Their B/I ratio
was 0.61 +/- 0.23. WB **isoforms** were 59 +/- 3% and their B/I
0.76 +/- 0.14. FB FSH **isoforms** were not detected. The whole
serum B/I ratio was 0.60 +/- 0.30. Similar results were obtained when
pooled sera was analysed: WB 43%, B/I 0.42; FB 57%, B/I 0.62. Whole
serum
B/I in pooled samples was 0.70. CONCLUSIONS: These results show that, in
normal women, circulating FSH bioactivity is associated with
isoforms with different oligosaccharide [correction of
oligosacharide] structures according to hormonal status. FSH in the
follicular phase has a higher degree of branching and a more complete
carbohydrate chain than the FSH secreted during the **menopause.**

L13   ANSWER 2 OF 4   MEDLINE
AN   96379946   MEDLINE
DN   96379946   PubMed ID: 8787959
TI   Undetectable luteinizing hormone levels using a monoclonal immunometric
      assay.
AU   Barbe F; Legagneur H; Watrin V; Klein M; Badonnel Y
CS   Service de Biologie Medicale, Maternite Regionale, Nancy, France.
※ SO JOURNAL OF ENDOCRINOLOGICAL INVESTIGATION, (1995 Nov) 18 (10) 806-8.
      Journal code: 7806594. ISSN: 0391-4097.
CY   Italy
DT   Journal; Article; (JOURNAL ARTICLE)
LA   English
FS   Priority Journals
EM   199610
ED   Entered STN: 19961106
      Last Updated on STN: 19961106
      Entered Medline: 19961021
AB   Previous studies have shown wide discrepancies among the results obtained
      with different immunometric assays. We present five cases (out of 4000
      women) whose plasma luteinizing hormone was not detected using a LH
      immunometric **assay** (LH Stratus Baxter) but was recognized by
      other kits. These cases concerned one 28-year-old woman presenting with
      infertility and four postmenopausal women. The LH Amerlite kit gave
      detectable but low results. The results obtained with the other kits
were
      > 7 IU/l. FSH levels were > 7 IU/l. In one case, sera were taken before

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Please give me a copy of the following references:

1. Creus et al., Clinical Endocrinology. 1996 Feb. 44 (2). pp. 181-189.
2. Barbe et al., Journal of Endocrinological Investigation. Nov. 1995. 18 (10). pp. 806-808.
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COMMENT

Undetectable luteinizing hormone levels using a monoclonal immunometric assay

F. Barbé*, H. Legagneur*, V. Watrin*, M. Klein**, and Y. Badonnel*

*Service de Biologie Médicale, Maternité Régionale, Nancy Cedex, **Clinique Médicale et Endocrinologique, Centre Hospitalier et Universitaire de Nancy, Hôpitaux de Brabois, Hôpital d'Adultes, Vandoeuvre-les-Nancy Cedex, France

ABSTRACT. Previous studies have shown wide discrepancies among the results obtained with different immunometric assays. We present five cases (out of 4000 women) whose plasma luteinizing hormone was not detected using a LH immunometric assay (LH Stratus Baxter) but was recognized by other kits. These cases concerned one 28-year-old woman presenting with infertility and four postmenopausal women. The LH Amerlite kit gave detectable but low results. The results obtained with the other kits were >7 IU/l. FSH levels were >7 IU/l. In one case, sera were taken before and after the menopause; differences between the LH results increased. Discrepancies among LH assays kits have been attributed to variation both in standard curve calibration and in epitope specificity of the kit monoclonal antibodies. The Baxter kit might misrecognize some isoforms present in postmenopausal women. The present data illustrate the potential false results with such immunoassays in routine clinical laboratory testing. When undetectable LH results are not clinically explained or when disparities between LH and FSH are observed, we suggest using a second methodology or a bioassay if necessary. Improvement in LH assays and standardization might resolve the problem of discrepancies between the LH results.

INTRODUCTION

Previous studies from our group (1) and from others (2-6) have shown wide discrepancies among the results obtained with different human luteinizing hormone (LH) assay kits. We report and discuss herein data concerning five cases (out of 4000 women) whose plasma LH was not detectable using our routine LH immunoenzymoassay (LH Stratus Baxter) but was recognized by other kits.

MATERIALS AND METHODS

This retrospective study over the past three years concerned 4000 nonpregnant women in investigations of hypogonadism, fertility problems, *in vitro* fertilization programs, polycystic ovarian disease

or menopause. Measurements of plasma LH and FSH were performed using our routine immunofluoroenzymologic assays, LH and FSH Stratus Baxter, Maurepas, France. In cases of disparity between LH and FSH concentrations, the Stratus results were re-tested and other immunoenzymoassays (Vidas LH, BioMérieux, Marcy l'Etoile, France; LH Amerlite, Kodak Clinical Diagnostics Ltd, Amersham Products, Les Ulis, France; SR1 LH, Serono Diagnostics, Rungis, France; Fertigenix LH-EASIA, Medgenix Diagnostics, Fleurus, Belgium; IMx LH, Abbott Diagnostic, Rungis, France), a radioimmunoassay (^{125}I-HLH Coatria, BioMérieux, Marcy l'Etoile, France) or a bioassay (Dufau method) were performed. The seven immunoassays all involved monoclonal antibodies and a sandwich methodology.

RESULTS

Five women (0,13%) had concentrations of LH that were not detectable or were close to the detection limit when assayed with the Baxter kit (Table 1). Using the LH Amerlite kit, the LH levels were measurable, but low. The LH results obtained with

Table 1 - *Comparative plasma LH results obtained in five women using 7 immunometric assays and 1 bioassay.*

Patient	Age yr		LH Stratus	Vidas LH	LH Armerlite	LH(IU/l) SR1 LH	Fertigenix LH-EASIA	IMx LH	^{125}I-HLH Coatria	Bioassay	FSH (IU/L)
A (GnRH test)	28	T0	0		1,7		20,6	25,0	37,0		7,0
		T15	0	21.2			>50	60,5			8,5
		T30	0				>50	87,9			10,2
		T60	0				>50	95,5		192,8	11,7
B	44		0,3	21,6	5,0						51,4
C	53		0,2	27,4	6,1						119,0
D	44		0	7,2	1,4			20,4			10,6
D	45		0	34,6		25,0					88,6
E	41		0	13,3		14,6					60,0

the other immunoassays were >7 IU/l. FSH results were >7 IU/l.

Case A was a 28-year-old woman presenting with infertility. Results of the GnRH test, with blood drawn at injection (T0) and 15, 30 and 60 min later (T15, T30, T60) showed a LH release by most of the kits, whereas the Baxter kit failed to recognize the presence of LH. The other kits and a bioassay provided high LH results. The four other cases (Cases B, C, D, E) were postmenopausal women (ages 41 to 53 years). It is noteworthy that the blood samples in case D were taken before and after her menopause; the woman presented wider differences between the LH results one year after the first determination, whereas FSH level increased.

DISCUSSION

Our results of undetectable LH are in agreement with those reported by others involving other methodologies (4, 7, 8), unpublished name kits (9) or unpublished patient data (1, 10). The three cases reported by Costagliola et al. (9) concerned a postmenopausal woman, a patient with polycystic ovary disease and another with renal failure (n=304 subjects with different pathologies or not, unpublished name kits). Those reported by Pettersson et al. (4) were five normal subjects (n=83 normal subjects) whose LH levels were undetectable using two immunometric assays involving monoclonal antibodies directed against the holomolecule (a non commercial immunofluorometric assay and the Enzymun-test LH, Boehringer, Mannheim). Gervasi et al. (8) using an immunoradiometric assay (LH IR-MA Immunotech) reported two cases, a post-

menopausal women and a premenopausal woman with infertility (n=155 subjects with different pathologies or not). No special LH isoform was characterized in the serum from the premenopausal woman, but a predominant amount of acidic isoforms. Billion (7) using an immunoradiometric assay (RIA-gnost hLH, Behring) reported three cases of zero LH levels in 32 out of 360 serial blood samples from 10 normal women.

Discrepancies among LH assays kits have been attributed to variation both in standard curve calibration and in epitope specificity of the kit monoclonal antibodies (9, 11). The LH Stratus Baxter kit uses, according to the manufacturer, monoclonal antibodies specific for the holohormone (anti-$\alpha\beta$) as labelled and coated probes. However, the exact specificity of the monoclonal antibodies differed in some cases with the specificity claimed by the manufacturers (11). The group of LH kits using anti-$\alpha\beta$ monoclonal antibodies either in solid (coated) or liquid (labelled) phase (12) or only in the liquid phase (9) has been reported to misrecognize circulating LH. Bugugnani (13) has indeed shown lower LH values using the Baxter kit compared to those obtained with IRMA BioMérieux (n=235 sera). In contrast to kit calibration, isoform misrecognition depends not only on the kit characteristics e.g. the pair of monoclonal antibodies, but also on the characteristics of the serum tested e.g. the panel of LH isoforms (9). Four cases (out of 5/4000) in our study are postmenopausal women. Especially in postmenopausal women acidic isoforms of LH are seen that are virtually absent in premenopausal women (12). The Baxter antibodies might misrecognize these acidic isoforms. The high specificity of the

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monoclonal antibodies could emphasize heterogeneity of LH molecular species (14), particularly in these cases with different proportions of LH isoforms. In the cases reported here, the LH Amerlite kit gave lower results compared with the other kits. This kit uses, according to the manufacturer, as labelled probes, anti β-LH monoclonal antibodies and as coated probes monoclonal antibodies specific for the holohormone (anti-αβ). Another explication of the undetectable LH levels has been described by Pettersson (15) who has characterized an immunologically anomalous luteinizing hormone genetic variant in a healthy women. Another hypothesis may involve the presence of cleavage products of LH β-subunit (16).

What are we measuring in gonadotrophin assays (14)? The present data from undetectable LH levels using monoclonal antibodies illustrate the potential false results with such immunoassays in routine clinical laboratory testing. When undetectable LH results are not clinically explained or when disparities between LH and FSH are observed, we suggest using a second methodology or a bioassay if necessary. Improvement in LH assays and standardization might resolve the problem of discrepancies between the LH results.

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and after the **menopause;** differences between the LH results
increased. Discrepancies among LH **assay** kits have been
attributed to variation both in standard curve calibration and in epitope
specificity of the kit monoclonal antibodies. The Baxter kit might
misrecognize some **isoforms** present in postmenopausal women. The
present data illustrate the potential false results with such
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not clinically explained or when disparities between LH and FSH are
observed, we suggest using a second methodology or a bioassay if
necessary. Improvement in LH assays and standardization might resolve
the
problem of discrepancies between the LH results.

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L13   ANSWER 3 OF 4   CAPLUS   COPYRIGHT 2003 ACS
AN    2001:747245   CAPLUS
DN    135:252338
TI    Test methods and devices for analyte isoforms
IN    Butlin, Lorraine Diane; Coley, John; Eida, Stephen James; Gani, Mohamed
      Mutwahar
PA    Unilever PLC, UK; Unilever N.V.
SO    Eur. Pat. Appl., 8 pp.
      CODEN: EPXXDW
DT    Patent
LA    English
FAN.CNT 1
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	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	EP 1143250	A2	20011010	EP 2001-303132	20010402
	R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO				
	JP 2001349892	A2	20011221	JP 2001-103658	20010402
	CA 2343488	AA	20011003	CA 2001-2343488	20010403
	US 2002042149	A1	20020411	US 2001-828624	20010403
PRAI	EP 2000-302810	A	20000403		

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AB    A method and test device for differentiating between states of an analyte
      that can exist in different forms, such as FSH (FSH). The method or test
      device uses two contemporaneous assays, the first of which does not
      differentiate between the two analyte states and the second of which
does,
      and the assay results are compared. A novel pair of anti-FSH
      monoclonal antibodies that can be used together in a sandwich-format
      assay to differentiate premenopausal and postmenopausal FSH
      samples is disclosed.
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L13   ANSWER 4 OF 4   CAPLUS   COPYRIGHT 2003 ACS
AN    2001:747244   CAPLUS
DN    135:252337
TI    Test methods and devices for analyte isoforms
IN    Butlin, Lorraine Diane; Coley, John; Eida, Stephen J.; Gani, Mohamed M.
PA    Unilever PLC, UK; Unilever N.V.
SO    Eur. Pat. Appl., 9 pp.
      CODEN: EPXXDW
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09/824,587

FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI EP 1143249	A2	20011010	EP 2001-303130	20010402

R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
        IE, SI, LT, LV, FI, RO

CA 2342683	AA	20011003	CA 2001-2342683	20010402
JP 2001349891	A2	20011221	JP 2001-103646	20010402
US 2002045273	A1	20020418	US 2001-824587	20010403
PRAI EP 2000-302811	A	20000403		

AB   A method and test device for differentiating between states of an analyte
      that can exist in different forms, such as FSH. The method or test
device
      uses a pair of specific binding agents, esp. monoclonal antibodies, in
two
      assays for the same analyte. The assays, applied to contemporaneous
      samples, differ from one another in format, one being a two step
      assay and the other being one step. A novel pair of anti-FSH
      monoclonal antibodies that can be used together in two such assays to
      differentiate pre-menopausal and post-menopausal FSH samples is
disclosed.

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US 20020045273A1

(54) **TEST METHODS AND DEVICES**

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Publication Classification

(57) **ABSTRACT**

A method and test device for differentiating between states of an analyte that can exist in different forms, such as follicle stimulating hormone (FSH). The method or test device uses a pair of specific binding agents, especially monoclonal antibodies, in two assays for the same analyte. The assays, applied to contemporaneous samples, differ from one another in format, one being a two step assay and the other being one step. A novel pair of anti-FSH monoclonal antibodies that can be used together in two such assays to differentiate pre-menopausal and post-menopausal FSH amples is disclosed.

self

TEST METHODS AND DEVICES

FIELD OF THE INVENTION

[0001] This invention relates to test methods and devices, and more particularly to methods for differentiating between states of an analyte that exists as various forms, e.g. isoforms.

BACKGROUND TO THE INVENTION

[0002] Tests are available, or have been proposed, which purport to provide clinically significant information about hormonal levels of relevance to the menopause. The principal hormone of interest is follicle stimulating hormone (FSH). The post-menopausal state has been associated with a rise in the level of circulating FSH. For this purpose tests have been developed to detect the level of FSH in body fluid samples such as blood and urine. These tests are intended to detect "total" FSH, in the sense that they do not discriminate between different isoforms of FSH.

[0003] These known tests are used by clinicians in recommending and monitoring hormone replacement therapy (HRT). As the menopause is also associated with a drop in the level of circulating estrogen metabolites, HRT normally involves administration of estrogen in order to reduce this deficit and counteract symptoms associated with the menopause.

[0004] Although it is known that FSH exists in various forms, the clinical significance of these in relation to conditions such as the menopause is poorly understood. The differing forms may be isoforms or glycoforms. However, the existence of these differing forms calls into question the soundness of "total" FSH tests as a basis for good clinical diagnosis.

[0005] There is a need for an improved method of monitoring gonadotrophin hormones, especially FSH, to provide more reliable diagnosis of menopausal conditions and to facilitate the prescription and regulation of HRT.

[0006] More generally, there is a need for a method for differentiating between states of an analyte that exists as a plurality of forms, especially when the nature and/or relative amounts of such forms present in a sample of the analyte may be of clinical significance. The forms may differ from one another in either physical characteristics (e.g. "isoforms" separable by charge) or chemical characteristics (e.g. "glycoforms" in the case of FSH or similar molecules), or indeed both.

GENERAL DESCRIPTION OF THE INVENTION

[0007] The invention provides a method for differentiating between two states of an analyte that exists in a plurality of forms, which states differ from one another in the nature and/or amount of one or more forms present therein, in which method a sample, or contemporaneous samples, containing the analyte are: (a) subjected to a two step specific binding assay utilising a first binding agent specific for the analyte and a labelled second binding agent specific for the analyte to provide a first test signal proportional to the amount of analyte present in the sample, wherein, in a first step of the two step assay, the analyte is contacted with the first binding agent to form a first binding agent/analyte complex, and in a second step of the two step assay, the first

binding agent/analyte complex is contacted with the labelled second binding agent, to form a first binding agent/analyte/second biding agent complex; and (b) said sample or samples are also subjected to a one step-specific binding assay utilising the same pair of analyte-specific binding agents, in which one step assay the analyte is contacted with both first and second binding agents substantially simultaneously, to form the first binding agent/analyte/second binding agent complex, to provide a second test signal proportional to the amount of analyte present in the test sample; and wherein at least one member of said pair of binding agents having a different specificity for each of said two states of said analyte, and the first test signal is compared to the second test signal.

[0008] The one step assay involves contacting the analyte with the first and second binding agents "substantially simultaneously". A short delay between the analyte coming into contact with one or other of the binding agents, of the matter of say 1-2 minutes (at room temperature) will not normally be critical. What is important is that the analyte does not have an opportunity to reach equilibrium, with respect to its binding to one of the binding agents, before coming into contact with the other binding agent. Clearly this depends on the kinetics of the reaction, which in turn depend on the identities of the analyte and binding partners involved, and the temperature. Accordingly the term "substantially simultaneously" as used herein should be construed as meaning that both binding agents should be contacted with the sample before 60%, (preferably before 70%, more preferably before 80%, and most preferably before 90%) of the analyte in the sample has become complexed with one of the binding agents.

[0009] Preferably, each member of said pair of binding agents has a different specificity for each of said two states of said analyte.

[0010] Desirably the specific binding agents comprise an antigen binding site of an immunoglobulin. One or both specific binding agents may advantageously be an antibody (e.g. an IgG or IgA molecule), especially a monoclonal antibody, or comprise any antigen binding portion thereof, such as Fv, Fab, scFv, bispecific antibodies, "diabodies" and the like, all of which are well known to those skilled in the art.

[0011] Conveniently, a combined test result may be expressed as a ratio of the two test signals. Optionally, the ratio of the two test signals is compared to a standard ratio for one or other of the two states to determine in which state the sample analyte exists.

[0012] The method of the invention is especially applicable when the analyte is a gonadotrophin, such as FSH.

[0013] It will be apparent to those skilled in the art that the first and second binding agents must not bind to identical sites on the analyte, so that a complex (the "first binding agent/analyte/second binding agent complex") may be formed in which both first and second binding agents are simultaneously bound to the analyte, in a "sandwich" type assay, well known to those skilled in the art.

[0014] In the two step assay, it is generally preferred that following performance of the first step, the first binding agent/analyte complex is separated from any excess analyte present in the sample prior to contacting the complex with

the labelled second binding agent, so as to prevent binding of the second binding agent to any uncomplexed analyte. This separation may be achieved in any suitable manner. For example, in one embodiment, in the first step of the assay the sample is incubated with a solid phase on which is immobilised the first binding agent, and thereafter following a washing step to remove unbound analyte, the solid phase is incubated with the labelled second binding agent.

[0015] In the one step assay the sample may be simultaneously incubated with a solid phase on which the first binding agent is immobilised and with the labelled binding agent in solution or suspension. Preferably, however in the one step assay the sample is simultaneously incubated with the first binding agent in solution or suspension and with the labelled second binding agent in solution or suspension, and the first binding agent is thereafter immobilised on a solid phase.

[0016] The solid phase may be any suitable solid support, such as a microtitre place, a membrane, a latex bead or the like.

[0017] As one option, immobilisation of the first binding agent on the solid phase is effected through a specific binding reaction, such as an avidin-biotin interaction, but numerous other specific interactions are known and could be employed (e.g. coating the solid phase with an Ig-specific antibody).

[0018] It will be apparent from the foregoing that it is an essential feature of the invention that at least one of the specific binding agents is labelled, to allow at least qualitative (preferably quantitative) detection of the formation of the first binding agent/analyte/second binding agent complex. Any conventional labelling method may be used (e.g. an enzyme label, fluorescent label, radio-label), but preferred labels include direct particulate labels, such as a gold sol or a coloured latex particle.

[0019] Generally it is the second binding agent which is labelled. However, it is possible that the first binding agent may also be labelled (for example, with biotin or some other label, to facilitate immobilisation on a solid phase, as outlined above). In such circumstances, it is desirable that the label on the first binding agent should not interact in any way with the detection system used in the assay to detect the label on the second binding agent. Conveniently this is arranged by ensuring that any label provided on the first binding agent is different to the label provided on the second biding agent.

[0020] In particular the invention provides a method of monitoring the hormonal status of an individual human female subject in which the contemporaneous tests are conducted repeatedly, i.e. at regular intervals such as every few weeks, to determine whether the gonadotrophin level and/or its character are changing in a manner which indicates entry into or departure from a menopausal state.

[0021] Another embodiment of the invention is an assay device for testing a body fluid sample obtained from a human subject (preferably a female), the device having a first analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable signal by means of a two step assay as described herein, and a second analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable

signal by means of a one step assay as described herein. Typically the assay signals will differ depending on whether the sample is derived from a pre-menopausal or post-menopausal subject.

[0022] In a further aspect, the invention provides a test kit for testing a body fluid sample obtained from a human subject (preferably a female), the kit comprising a first analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable signal by means of a two step assay as described herein, and a second analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable signal by means of a one step assay as described herein, together with instructions for use in the method of the invention. In one embodiment, the first and second analyte-responsive signal-producing means are provided on a single test device. In an alternative embodiment, the first and second signal-producing means are provided on respective first and second test devices.

[0023] Each readable signal can be caused by the binding in a detection zone of a specific binding agent labelled with a direct particulate label, such as a gold sol or coloured latex particle. Alternatively, other signal-producing labels can be used, for example enzyme labels, fluorescent labels or radio-labels.

[0024] The contemporaneous tests of the invention can be conducted repeatedly, generally at an interval of at least a week, to monitor the effectiveness of a course of HRT.

[0025] Although FSH is the preferred analyte for use in accordance with the invention, other members of the gonadotrophin family can be used. These include human chorionic gonadotrophin (hCG), luteinizing hormone (LH) and thyroid stimulating hormone (TSH). All of these gonadotrophins are glycopeptides. Their principal structure comprises two peptide chains. One peptide chain, known as the alpha chain, is common to all members of the family. The other peptide change, known as the beta chain, differs in each molecule. In addition, each molecule contains glycoprotein side chains. The detailed structure of these molecules is not completely understood. However it is believed that variations in the composition of the glycoprotein side chains give rise to different forms ("glycoforms") of each molecule. Those skilled in the art will appreciate that differences in the chemical properties of the glycoprotein side chains may also influence the physical properties (e.g. charge) of the overall molecule, such that different glycoforms may also constitute different isoforms. Thus, in the case of FSH for example, on present scientific knowledge it is believed that the alpha and beta peptide chains are the same in all FSH forms, but subtle differences occur in the glycoprotein side chains. It is believed that the relative proportions of the forms of FSH existing in the menopause state are different from those in the pre-menopause state.

[0026] Prior to this invention it was not appreciated that a combination of specific binding assays could be developed which would differentiate between the FSH forms, to an extent sufficient to enable worthwhile detection of a menopausal state to be achieved, or that by using two different formats together an assay could provide enhanced differentiation.

[0027] In a preferred embodiment of the invention both assays are of the sandwich format. Each assay therefore

requires two specific binding agents (e.g. antibodies), one preferably directed against the alpha chain and the other preferably against the beta chain of the FSH molecule. The two antibodies must be different. In a preferred embodiment the invention uses two sandwich-format immunoassays for FSH, one a two step assay and the other a one step assay, in which the antibodies are directed against the alpha and beta peptide chains of the molecule, but are exhibiting differences in specificity for certain forms of FSH caused by subtle changes in the glycoprotein side chains.

[0028] Antibody pairs appropriate for use in the invention can be identified by screening a range of anti-FSH antibody pairs against FSH samples obtained from pre-menopausal and post menopausal women.

[0029] In order to provide a source of antibodies from which to select an antibody pair which under the circumstances of the method of the invention differentiate between analyte forms, it is desirable, although not essential, to raise a panel of antibodies against the analyte forms in question. This can be done by routine hybridoma technology. Alternatively, immunoglobulin-producing bacteriophage libraries may be screened.

[0030] A particular aspect of the invention in relation to its application to the analysis of FSH samples is a pair of novel anti-FSH monoclonal antibodies that distinguish between pre-menopausal and post-menopausal FSH samples. Two murine hybridoma cell lines each expressing one of these novel monoclonal antibodies have been deposited in accordance with the provisions of the Budapest Treaty 1977 in the European Collection of Cell Cultures (ECACC, Centre for Applied Microbiology & Research, Salisbury, Wiltshire SP4 0JG, UK)as follows:

[0031] a) Balb/c murine hybridoma clone "4813.2" expressing an anti-beta-FSH monoclonal antibody: ECACC 00032004; and

[0032] c) Balb/c murine hybridoma clone "4882.1" expressing an anti-alpha-FSH monoclonal antibody: ECACC 00032005, (both deposited on Mar. 20, 2000).

[0033] The invention includes the use of either or both of the anti-FSH monoclonal antibodies as expressed by these deposited cell lines, in a method or analytical test device as set forth herein.

[0034] The methods of the invention may be performed using any suitable sample, generally samples of body fluid from a subject. The body fluid may be any suitable body fluid, such as blood, serum, plasma, sweat, tears, crevicular fluid and the like. Most conveniently the sample is a sample of urine, which can easily be obtained without performing any invasive procedure.

[0035] In practice the two assays should be performed on the same clinical sample, sub-divided if necessary, or on two samples obtained from the same individual subject at more or less the same time (i.e. on the same day, and preferably in the same hour), so that the two assays give results that can fairly be compared with each other. It is in this sense that we regard the assays as being contemporaneous. The results of the two contemporaneous assays are compared to determine whether a menopausal state exists.

[0036] In one embodiment the test results can be interpreted on a qualitative or semi-quantitative basis, for example by eye if the two assays give rise to visible test readings which can be interpreted readily, for example through differences in colour or colour intensity. If necessary this visual determination can be aided by the provision of a reference standard. The two assays can be configured to aid visual assessment.

[0037] For more accurate diagnosis of menopausal conditions it may be appropriate for the assay results to be determined numerically. This will usually require a sophisticated reading system, such as by optical transmission or reflectance and which is amenable to measuring small changes in signal intensity and relating these to FSH concentrations. In this situation it may be appropriate to determine the numerical ratio of the signals of the first and second assays. A significant change in this ratio can indicate transition from a pre-menopausal to a post-menopausal state, or vice-versa. Thus the results from a series of contemporaneous tests performed, for example, every few weeks, can be collated and any change in the observed signal ratio used to diagnose a change in condition.

[0038] For the purposes of HRT monitoring, the HRT treatment, either in terms of the therapeutic product used or its dose level, can be modulated to maintain the ratio value from successive contemporaneous tests at a pre-determined level, for example.

[0039] Test devices using the assays of the invention can be provided for home use or for use in clinics or doctors' offices. Alternatively laboratory-style assays can be used. Preferred assay formats involve the single step format as described, for example, in EP-A-291194. These assays can be used if desired in combination with an electronic reader, for example as described in WO 95/13531. In this instance preferably the electronic reader has an information downloading facility, e.g. by means of a transferable datacard ("smart card"), from which a user, e.g. clinician, can transfer data to a computer during consultation with the patient, in order that stored information from repeated tests can be interpreted properly for diagnostic purposes. The computer can include programmed information that assists the clinician in establishing an appropriate HRT treatment for the individual subject.

[0040] Generally, the method of the invention involves the use of one pair of specific binding agents in two contemporaneous assays differing in format.

[0041] The following example illustrates aspects of the invention in greater detail.

EXAMPLE

[0042] 1. Raising Anti-FSH Monoclonal Antibodies

[0043] Balb/c mice were immunised with human FSH preparations, derived from urine and pituitary sources, purified by immuno-affinity prior to immunisation. Monoclonal antibodies were produced from the immunised mice using conventional cloning techniques, by fusing spleen cells with SP2/Ag14 cells as the immortal partner.

[0044] The subunit specificity of the anti-FSH monoclonal antibodies were assigned by means of anti-alpha subunit antibodies cross reactive with LH, TSH and hCG.

[0045] 2. Identification of Anti-FSH Antibody Pairs with Fertile State Bias

[0046] a) A panel of seven human urine samples were used to screen various antibody pairs. The samples from young fertile women (under 35 years of age with regular menstrual cycles) were taken at the (1) early follicular, (2)mid-follicular, (3)ovulation, (4)mid-luteal and (5)late luteal phases of menstrual cycles. For each fertile phase pooled samples from two individuals were used. The phase of the menstrual cycle was determined retrospectively by the urinary profiles of the hormones FSH,LH, E3G and P3G. In addition to the fertile samples, two post menopausal urine samples were used (6) 1 month before commencement of HRT treatment and (7)one taken at least one month after HRT treatment began.

[0047] For use in the antibody screen the urine panel was normalised based on FSH concentration estimates. The FSH concentration estimates were obtained using commercially available anti-FSH monoclonal antibodies (Clone No's. 6601 and 6602 from Medix Biochemica, Finland) in a sandwich-format ELISA assay. Samples were normalised by being concentrated using centrifuge filtration.

[0048] b) Screening procedure

[0049] The following standard buffer solutions were used:

[0050] PBSTA:

[0051] Phosphate buffered saline

[0052] 0.01M Phosphate pH 7.2

[0053] 0.9% Sodium chloride

[0054] 0.15% Tween 20

[0055] 0.02% Sodium azide

[0056] PBSA:

[0057] As above, no Tween 20

[0058] PBS:

[0059] As above, no Tween 20 or sodium azide

[0060] 1) FSH antibodies for screening were prepared to concentrations of 2.5 μg/ml in 0.2M sodium carbonate buffer pH 8.0.

[0061] 2) 20011 of the antibody dilutions were added to wells in High binding Greiner 96-well microtitre plates, which were then incubated overnight at 37° C.

[0062] 3) The plates were washed three times in PBSTA.

[0063] 4) 100 μl of 0.38M Tris was added to into all of the plate wells, except the blanking wells to which PBSTA was added. 100 μl of each urine sample from the screening panel (see paragraph a) were added at 15.3 mIU/ml (based on the 6602/6601 assay estimates) to triplicate wells for each antibody/conjugate pairing.

[0064] 5) The plates were incubated for 1 hour at room temperature.

[0065] 6) Step 3 was repeated.

[0066] 7) 200 μl of an optimum dilution in PBSTA of alkaline-phosphatase conjugated anti-beta FSH subunit antibody was added to wells sensitised with anti-alpha FSH subunit capture antibodies. 200 μl of an optimum dilution in PBSTA of alkaline-phosphatase conjugated anti-alpha FSH subunit antibody was added to wells sensitised with anti-beta FSH subunit capture antibodies.

[0067] 8) As a control in each screening run the urine panel was tested with a reference assay (the "Medix Assay") using Medix Biochemica clone No. 6601 conjugate paired with Medix Biochemica clone No.6602 as the capture antibody.

[0068] 9) Step 5 was repeated.

[0069] 10) Step 3 was repeated.

[0070] 11) 200 μl of DEAE substrate was pipetted into all wells of all plates.

[0071] 12) Step 5 was repeated.

[0072] 13) The plates were read on a Dynatech plate reader at 405 nm after 1 hour 30 minutes incubation.

[0073] 14) The mean value of the triplicates were then calculated, and compared to the Medix assay O.D. values.

[0074] This allowed antibody pairs showing bias in sample recognition relative to the Medix assay to be identified.

[0075] The two hydridoma cell lines referred to earlier, now deposited with the ECACC, were selected using this procedure.

[0076] 3. Use of Contemporaneous two Step/One Step FSH Assays for Menopause Confirmation

[0077] The method described in section 4 below was used to test 8 consecutive daily urine samples (around mid-cycle) taken from one fertile woman, and 9 consecutive daily urine samples from one post-menopausal woman.

[0078] The FSH concentration in each sample was measured by the two step and one step assays. The ratios obtained are shown in the Table below, and clearly differentiate between fertile and post-menopausal states.

[0079] 4. Test Methods

[0080] Both assays were carried out in a "Delfia"[RTM] Time Resolved Fluoroimmunoassay, supplied by Perkin Elmer Life Sciences, using the standard reagents and buffers as supplied, except where indicated.

[0081] a) Two Step Assay

[0082] 1) FluoroNunc 96-well microtitre plates were sensitised with 200 μl of 5 μg.ml 4813.2 antibody in PBSA coating buffer overnight at 4° C.

[0083] 2) 4813.2-sensitised plates were washed 2 times with Wash Concentrate (Catalogue No. 1244-114).

[0084] 3) 200 μl of a blocking buffer was added to each well, and incubated for 1 hour at room temperature with shaking. The blocking buffer was the coating buffer plus 2% BSA.

[0085] 4) Step 2 was repeated.

[0086] 5) 50 μl of FSH standard or urine sample and 200 μl Assay Buffer was added per well.

[0087] 6) Incubated for 6 hours at room temperature with shaking.

[0088] 7) Wash 3 times with Wash Concentrate.

[0089] 8) 200 μl of Europium-labelled 4882.1 antibody in Assay Buffer (atalogue No. 1244-111) added, and incubated for 1 hour at room temperature with shaking. The labelling was conducted using a commercial Europium labelling kit from Perkin Elmer, according to the manufacturer's instructions.

[0090] 9) Step 7 was repeated.

[0091] 10) 200 μl Enhancement Solution (Catalogue No. 1244-105) added to each well.

[0092] 11) Incubation for 5 minutes at room temperature with shaking.

[0093] 12) Read result.

[0094] b) One Step Assay

[0095] 1) Proceed through steps (1) to (4) of assay (a).

[0096] 2) Add 200 μl FSH standard or urine sample, and 200 μl Eu-labelled 4882.1 antibody, and incubate for 2 hours at room temperature with shaking.

[0097] 3) Proceed through steps (9) to (12) of assay (a).

Individual and Sample Day	Result Ratio of two step to one step Assays
Fertile	
D14	3.5
D15	3.8
D16	3.8
D17	4.0
D18	3.8
D19	2.9
D20	3.4
D21	3.1
Post-Menopausal	
D10	1.2
D11	1.3
D12	1.2
D13	1.8
D14	1.3
D16	1.3
D17	1.3
D18	1.3
D19	1.5

1. A method for differentiating between two states of an analyte that exists in a plurality of forms, which states differ from one another in the nature and/or amount of one or more forms present therein, in which method a sample, or contemporaneous samples, containing the analyte are: (a) subjected to a two step specific binding assay utilising a first binding agent specific for the analyte and a labelled second binding agent specific for the analyte to provide a first test signal proportional to the amount of analyte present in the sample, wherein, in a first step of the two step assay, the analyte is contacted with the first binding agent to form a first binding agent/analyte complex, and in a second step of the two step assay, the first binding agent/analyte complex is contacted with the labelled second binding agent, to form a first binding agent/analyte/second biding agent complex; and (b) said sample or samples are also subjected to a one step specific binding assay utilising the same pair of analyte-specific binding agents, in which one step assay the analyte is contacted with both first and second binding agents substantially simultaneously, to form the first binding agent/analyte/second binding agent complex, to provide a second test signal proportional to the amount of analyte present in the test sample; and wherein at least one member of said pair of binding agents having a different specificity for each of said two states of said analyte, and the first test signal is compared to the second test signal.

2. A method according to claim 1, wherein each of said first and second binding agents has a different specificity for each of said two states of said analyte.

3. A method according to claim 1 or claim 2, wherein a combined test result is expressed as a ratio of the two test signals.

4. A method according to claim 3, wherein the ratio of the two test signals is compared to a standard ratio for one or other of the two states to determine in which state the sample analyte exists.

5. A method according to any one of the preceding claims, wherein the analyte is a gonadotrophin.

6. A method according to claim 5, wherein the analyte is follicle stimulating hormone (FSH).

7. A method according to any one of the preceding claims, wherein both first and second binding agents are antibodies.

8. A method according to claim 7, wherein each binding agent is a monoclonal antibody.

9. A method according to any one of the preceding claims, wherein in the two step assay the sample is incubated with a solid phase on which is immobilised the first binding agent, and thereafter following a step to remove unbound analyte the solid phase is incubated with the labelled second binding agent.

10. A method according to any one of the preceding claims, wherein in the one step assay the sample is simultaneously incubated with a solid phase on which the first binding agent is immobilised and with the labelled second binding agent in solution or suspension.

11. A method according to any one of claims 1-9, wherein in the one step assay the sample is simultaneously incubated with the first binding agent in solution or suspension and with the labelled second binding agent in solution or suspension, and the first binding agent is thereafter immobilised on a solid phase.

12. A method according to claim 10 or 11, wherein immobilisation of the first binding agent on the solid phase is effected through a specific binding reaction.

13. A method according to claim 12, wherein the specific binding reaction is an avidin-biotin interaction.

14. A method for differentiating between an FSH sample indicative of a present or impending fertile status of the human ovulation cycle and an FSH sample indicative of a present or impending infertile status of the human ovulation cycle, substantially as hereinbefore described.

15. An anti-FSH monoclonal antibody as expressed by hybridoma cell line ECACC 00032004.

16. An anti-FSH monoclonal antibody as expressed by hybridoma cell line ECACC 00032005.

17. A method according to any one of claims 1 to 14, wherein the labelled second binding agent is an antibody as claimed in claim 15 and the first binding agent is an antibody as claimed in claim 16.

18. A test device for testing a body fluid sample obtained from a human subject, the device comprising a first analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable signal by means of a two step assay as described herein, and a second analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable signal by means of a one step assay.

19. A test kit comprising a test device according to claim 18, and instructions for use in a method according to any one of claims **1-14** or claim 17.

20. A test kit for performing a method according to any one of claims **1-14** or claim 17, the kit comprising a first test device having analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable signal by means of a two step assay, and a second test device having second analyte-responsive (preferably gonadotrophin-responsive) signal-producing means that provides a readable signal by means of a one step assay.

* * * * *

L13   ANSWER 1 OF 4   MEDLINE
TI   FSH **isoforms:** bio and immuno-activities in post-menopausal and
      normal menstruating women.
AB   . . .   The full expression of gonadotrophin biological activity depends
      on the gonadotrophin carbohydrate component. Our aim was to study serum
      FSH **isoforms** present in the follicular phase (FPS) and in the
      menopause (PMS) since the endocrine status may influence the
      structure of incorporated oligosaccharides. SUBJECTS: Ten healthy
      post-menopausal women (age range 53-68). . .   20-28) in the follicular
      phase of their menstrual cycle were studied. MEASUREMENTS: Bio and
immuno
      FSH-activities (Sertoli cell aromatase induction **assay** and RIA,
      respectively) were determined in separated **isoforms** after
      concanavalin A chromatography. Isolated **isoforms** were: UB,
      unbound; WB, weakly bound and FB, firmly bound to the lectin. RESULTS:
      PMS showed two groups of immuno and bio-active FSH **isoforms:** WB,
      bearing biantennary galactosylated type and FB, bearing high mannose or
      hybrid type oligosaccharides. Immuno and bio-active FSH were not
detected
      in the UB fractions. WB **isoforms** represented 82 +/- 6% of the
      total bioactivity recovered in samples analysed individually; their B/I
      ratio was 0.85 +/- 0.20. FB **isoforms** were 18 +/- 6%; their B/I
      ratio was 3.27 +/- 0.60. Whole serum B/I ratio was 1.20 +/- 0.30.
      Similar. . . B/I: 0.82; FB: 23%, B/I: 3.75. Whole serum B/I in pooled
      samples was 1.10. FPS showed a different pattern. UB **isoforms,**
      bearing triantennary or bisecting oligosaccharides, were 41 +/- 3% of the
      total bioactivity recovered in samples analysed individually. Their B/I
      ratio was 0.61 +/- 0.23. WB **isoforms** were 59 +/- 3% and their
      B/I 0.76 +/- 0.14. FB FSH **isoforms** were not detected. The
      whole serum B/I ratio was 0.60 +/- 0.30. Similar results were obtained
      when pooled sera was. . . B/I in pooled samples was 0.70.
CONCLUSIONS:
      These results show that, in normal women, circulating FSH bioactivity is
      associated with **isoforms** with different oligosaccharide
      [correction of oligosacharide] structures according to hormonal status.
      FSH in the follicular phase has a higher degree of branching and a more
      complete carbohydrate chain than the FSH secreted during the
      menopause.
CT   Check Tags: Female; Human; Male; Support, Non-U.S. Gov't
       Adult
       Aged
       Aromatase: ME, metabolism
         Biological Assay
       Chromatography, Gel
         Follicle Stimulating Hormone: BL, blood
        ***Follicle Stimulating Hormone: ME, metabolism**
      *Follicular Phase: ME, metabolism
       Isomerism
       Middle Age
      *Postmenopause: ME, metabolism
       Radioimmunoassay
       Sertoli Cells: DE, drug effects

L4   ANSWER 4 OF 8 CAPLUS   COPYRIGHT 2003 ACS
AN   1999:799609 CAPLUS
DN   132:119488
TI   Comparison of immunoreactivity of five human cardiac troponin I assays
      toward free and complexed forms of the antigen: implications for
      assay discordance
AU   Datta, Pradip; Foster, Kimberley; Dasgupta, Amitava
CS   Bayer Diagnostics, E. Walpole, MA, 02032, USA
SO   Clinical Chemistry (Washington, D. C.) (1999), 45(12), 2266-2269
      CODEN: CLCHAU; ISSN: 0009-9147
PB   American Association for Clinical Chemistry
DT   Journal
LA   English
AB   The cardiac **isoforms** of TNI (cTNI) and troponin T (TnT) are
      structurally different from the corresponding skeletal **isoforms**,
      and therefore they have recently established themselves as biochem.
      markers of myocardial damage. The currently available cTnI assays
produce
      differing results. We explored the possibility that the sera from
      patients with different pathol. conditions may contain different
      isoform distributions of cTnI, thus generating discordant results
      among assays that recognize the **isoforms** differently. Here we
      report the immunoreactivity of five commonly used com. cTnI immunoassays
      (Bayer ACS:180, Dade Stratus, Beckman Access, Behring OPUS, and Abbott
      AxSYM) toward the two major forms of the **analyte**: free cTnI and
      its binary complex, IC. We think that the differential immunoreactivity
      of cTnI assays to its **isoforms** may explain some cases of
      assay discordance. A possible example can be found in a method
      comparison study with samples from many categories of cardiac patients.
      When the Stratus, OPUS, and ACCESS **assay** results for 138 such
      samples were compared, a poorer linear regression coeff. (r = 0.774) was
      found between Stratus and ACCESS than between Stratus and OPUS (r = 0.92)
      or OPUS and ACCESS (r = 0.90). Our finding that the order of
equimolarity
      between these three assays is ACCESS (most equimolar) > OPUS > Stratus
      (least equimolar) may explain those data. An **assay** that detects
      both forms of cTnI equally well could be an advantage over a method that
      detects only one form of cTnI because the presence of any form of cTnI in
      serum is indicative of cardiac damage.
RE.CNT 18   THERE ARE 18 CITED REFERENCES AVAILABLE FOR THIS RECORD
              ALL CITATIONS AVAILABLE IN THE RE FORMAT

STIC-ILL

NPL

Please give me a copy of the following references:

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3. Span et al., International Journal of Biological Markers. 2000. 15(2), pp. 184-191.
4. Datta et al., Clinical Chemistry (Washington, d.C.) 1999. 45 (12), pp. 2266-2269
5. Smith et al., Infection and Immunity. 1994. 62 (12) pp. 5470-5476.

Thank You
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1

found in benign breast diseases (25). In addition, the presence of PSA (in particular in the uncomplexed form) in NAF is in accordance with the high concentration of enzymes, proteases, and some biochemical analytes that may be prone to metabolic alterations in ductal epithelial cells of women with breast diseases (11). In another study we described at the electron microscopic level the epithelial cells present in NAFs, giving evidence for highly metabolizing cells (Malatesta et al., submitted for publication). Accordingly, the intense anti-PSA labeling on ribosomes, sites of protein synthesis, strongly suggests that NAF cells are able to synthesize this protease; moreover, the presence of some labeling along the cell surface could indicate a secretory activity. The small vacuoles containing PSA could represent storage/complexation sites of this protease, although we cannot exclude that they can be removed by exocytosis. Therefore, breast epithelial cells present in NAFs could be a candidate source of PSA found in nipple aspirate extracellular fluid; similarly, in type I breast gross cysts, the apocrine cells occurring in the cystic fluid are responsible for PSA production and secretion (24).

The production and secretion of several proteases (including PSA) and biologically active compounds by female breast apocrine cells in association with a lack of physiologic control of the secretion/reabsorption mechanism (11) and/or with prolonged exposure to several biologically active compounds through the autocrine/paracrine mechanism (13) could, with age, make the highly metabolizing apocrine cells prone to premalignant transformation (26). However, the clinical significance of the PSA molecular forms and of its production/secretion by breast epithelial cells with respect to breast cancer risk should be evaluated cautiously (17, 18).

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Comparison of Immunoreactivity of Five Human Cardiac Troponin I Assays toward Free and Complexed Forms of the Antigen: Implications for Assay Discordance, *Pradip Datta,*[1]* *Kimberley Foster,*[1] *and Amitava Dasgupta*[2] ([1] Bayer Diagnostics, 333 Conney St., E. Walpole, MA 02032; [2] University of Texas-Houston, Houston, TX 77030; * author for correspondence: fax 508-660-4591, e-mail pradip.datta@chirondiag.com)

Troponin, consisting of three components, troponin C (TnC), troponin I (TnI), and troponin T (TnT), is a major component of the structural proteins involved in striated and cardiac muscle contraction (1, 2). TnI and TnC bind tightly to each other in the presence of Ca^{2+} with an association constant, K_a, of ~10^8-10^9 L/mol (1–3). TnT binds to both TnC and TnI, although less weakly than the binding between TnC and TnI. The cardiac isoforms of TnI (cTnI) and TnT are structurally different from the corresponding skeletal isoforms, and therefore they have recently established themselves as biochemical markers of myocardial damage (4–7).

The currently available cTnI assays produce differing

results *(8–10)*. One important reason is that the assays may differ in their responses to the various isoforms of cTnI present in circulation or in biochemical preparations. In addition to "free" cTnI (I) and its binary and ternary complexes with TnC and TnT (IC or ICT) *(8, 11, 12)*, cTnI may exist in phosphorylated *(13)*, oxidized *(14)*, and proteolytically degraded *(8, 11, 12)* forms. The latter modified forms of cTnI also may exist as binary or ternary complexes. All of these forms may have different recognition patterns in different immunoassays. The predominant form of cTnI in acute myocardial infarction (AMI) patients is the binary complex IC *(8, 11)*. Samples treated with a Ca^{2+} chelator such as EDTA would contain mostly free cTnI because chelation of Ca^{2+} disrupts IC and ICT complexes.

We explored the possibility that the sera from patients with different pathological conditions may contain different isoform distributions of cTnI, thus generating discordant results among assays that recognize the isoforms differently. Here we report the immunoreactivity of five commonly used commercial cTnI immunoassays (Bayer ACS:180®, Dade Stratus®, Beckman Access®, Behring OPUS®, and Abbott AxSYM®) toward the two major forms of the analyte: free cTnI and its binary complex, IC.

Free cTnI (I) and its binary complex (IC), both isolated from human heart, were obtained from Scripps Laboratories. Their concentrations, determined by protein assay (Bio-Rad), were provided by the vendor and then converted to molar cTnI concentrations, using molecular weights of 21 000 and 35 000 for I and IC, respectively. Normal human serum, obtained from Scantibodies, tested negative for cTnI in all five cTnI assays.

Buffered stock solutions of both I and IC, as obtained from the vendor, and the serum were mixed to produce 0, 5, 10, and 20 μg/L solutions of cTnI, which were analyzed by five cTnI immunoassays: ACS:180, Stratus, AxSYM,

OPUS, and ACCESS. Manufacturer-suggested assay protocols and platforms were used for all assays. All five assays use a "sandwich" method, where the analyte is sandwiched between capture and label antibodies, and thus the generated signal is directly proportional to the concentration of TnI in the sample. Details of the assays are in listed in Table 1.

When the results of the methods listed in Table 1 (*y*) were compared with the Stratus (*x*), the linear correlation slopes were 0.14 for ACCESS, 1.13 for ACS:180, 1.43 for OPUS, and 3.42–5.14 for AxSYM. We calculated the OPUS (*y*)/Stratus (*x*) slope as the ratio of slopes for (ACS:180/Stratus) and (ACS:180/OPUS). The two different slopes of AxSYM (*y*) vs Stratus (*x*) were obtained from different studies: 3.42 from the package insert of the AxSYM kit, and 5.14, from our own studies *(15)*. The AMI cutoff values varied in slightly different order: ACCESS (0.15 μg/L) < ACS:180 ~ Stratus (1.5 μg/L) < AxSYM ~ OPUS (2.0 μg/L). The ACCESS and AxSYM assays differed by a factor of as much as 36-fold (5.14/0.14). The other three assays agreed more closely (± 25%). Various survey data for cTnI also indicate similar differences among the assay results *(16)*. Many of the difference are probably contributed by standardization. As noted by other researchers *(8–10)*, such interassay differences underscore the urgent need for universal standardization for this important analyte.

Fig. 1 presents the comparison of immunoreactivity of the five assays to IC (Fig. 1A) and I (Fig. 1B). The assays have different orders of immunoreactivity toward free cTnI and IC complex: ACCESS < Stratus < ACS:180 < OPUS < AxSYM for IC; and Stratus < ACCESS < OPUS < ACS:180 < AxSYM for I. The order of immunoreactivity for IC was similar to the order of their responses in the method comparison study (Table 1). However, whereas the ACS:180, ACCESS, and AxSYM cTnI assay

Table 1. Comparison of the five cTnI immunoassays used in this study.

	ACS:180	Stratus	AxSYM	OPUS	ACCESS
Format/label type	Sandwich/ chemiluminescent	Sandwich/ fluorescent	Sandwich/microparticle enzyme-fluorescent	Sandwich/ fluorescent	Sandwich/ chemiluminescent
Manufacturer	Bayer Corp. Diagnostics Division Walpole, MA	Dade Behring Miami, FL	Abbott Diagnostics Abbott Park, IL	Dade Behring Westwood, MA	Beckman Chaska, MN
Platform run on:	ACS:180 analyzer	Stratus II analyzer	AxSYM analyzer	OPUS Magnum analyzer	ACCESS analyzer
Capture antibody	Two different mouse monoclonal antibodies	Mouse monoclonal antibody[a]	Mouse monoclonal antibody	Goat polyclonal antibody[a]	Mouse monoclonal antibody[a]
Label antibody	Goat polyclonal antibody	Mouse monoclonal antibody	Goat polyclonal antibody	Goat polyclonal antibody	Mouse monoclonal antibody
Sample size, μL	100	200	220	250	50
Range, μg/L	0.15–50	0.3–50	0.3–50	0.5–150	0.03–50
Cutoff for AMI, μg/L	1.5	1.5	2.0	2.0	0.15
Method comparison slope (*y/x*)	1.13[b]	3.42[c]	0.22[c]	0.79[e]	0.14[d]
	(ACS:180/Stratus)	(AxSYM/Stratus)	(ACS:180/AxSYM)	(ACS:180/OPUS)	(ACCESS/Stratus)

[a] The capture antibody is different from the label antibody, recognizing different sites on the cTnI molecule.

[b–d] From the package inserts for the kits; [b] ACS:180; [c] AxSYM; and [d] ACCESS.
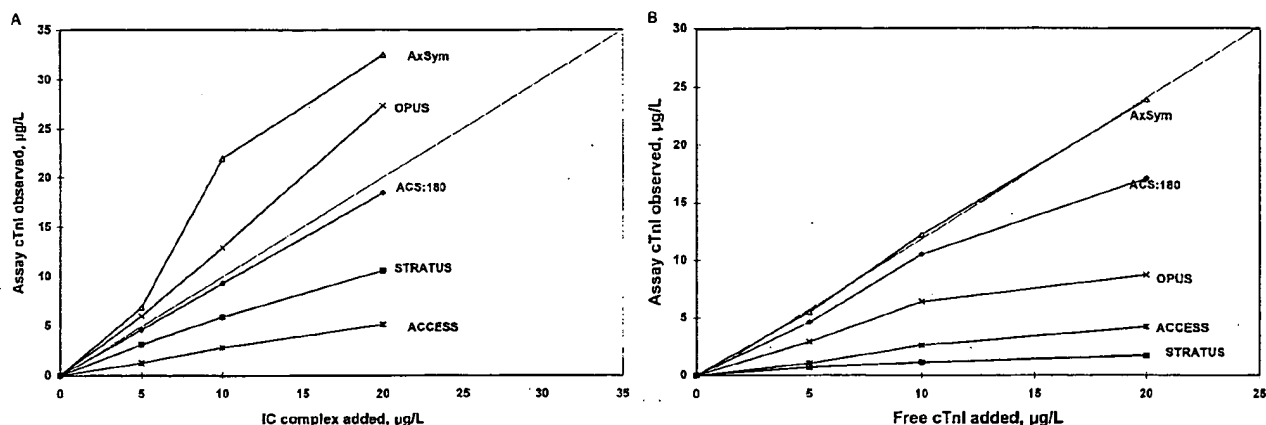
[e] Dasgupta et al. *(15)*.

Fig. 1. Results of five different cTnI assays compared for serum samples supplemented with binary IC complex (A) or free cTnI (B).

In A and B, the solid line is the line of identity. (A), the ACS:180 assay is closest to the line of identity, AxSYM and OPUS are above the line of identity, and ACCESS and Stratus are below the line of identity. The linear correlation slopes of the five assay results with respect to the added cTnI concentrations are: ACS:180, 0.92 (r = 0.999); Stratus, 0.53 (r = 0.998); AxSYM, 1.68 (r = 0.978); OPUS, 1.37 (r = 0.999); and ACCESS, 0.26 (r = 0.999). (B), the ACS:180 assay is closest to the line of identity, AxSYM is above the line, and the other assays are below the line of identity. The linear correlation slopes of the five assay results with respect to the added cTnI concentrations are: ACS:180, 0.86 (r = 0.992); Stratus, 0.08 (r = 0.978); AxSYM, 1.20 (r = 0.999); OPUS, 0.43 (r = 0.968); and ACCESS, 0.21 (r = 0.990).

responses to I remained similar to their responses to IC, the Stratus and OPUS assays behaved quite differently to I than to IC, producing responses to I that were 6.41 and 3.19 times less than IC (as determined the ratio of slopes, IC/I; Fig. 1). The ACS:180 cTnI assay showed the best "equimolarity" (i.e., the ability to recognize I and IC equally well), with a IC/I slope ratio of 1.07. The slope ratios for the ACCESS and AxSYM assays were 1.23 and 1.4, respectively.

Our observations agree well with those of Wu et al. (8) who compared nine different immunoassays for cTnI toward synthetically prepared oxidized or reduced forms of I or IC. When they compared the more common oxidized forms of IC and I, six assays in their study were approximately equimolar, two others responded to IC better than to I by ~1.5-fold, and one assay responded to IC ~3.2-fold more than to I.

Because the binary IC complexed form of cTnI is believed to account for ≥90% of cTnI in the serum of most AMI patients (8, 11, 12), the ACS:180 and Stratus assays show close agreement among most samples (Table 1). However, if the complexed form of cTnI is released into the circulation only after extensive damage to myocytes-sand the subsequent necrosis of cardiac muscle, the appearance of IC or ICT complexes in the circulation could take several hours to days, thus delaying the confirmation of AMI diagnosis. On the other hand, 5–6% of the total cardiac cTnI exists as a free form in the cytoplasm (17). If the free cTnI is released faster than IC complex from damaged myocytes, initial cTnI concentrations may contain a higher percentage of free cTnI. These samples may show a higher frequency of discordance between the equimolar and nonequimolar cTnI assays.

We think that the differential immunoreactivity of cTnI assays to its isoforms may explain some cases of assay discordance. A possible example can be found in a

method comparison study with samples from many categories of cardiac patients (18). When the Stratus, OPUS, and ACCESS assay results for 138 such samples were compared, a poorer linear regression coefficient (r = 0.774) was found between Stratus and ACCESS than between Stratus and OPUS (r = 0.92) or OPUS and ACCESS (r = 0.90) (18). Our finding that the order of equimolarity between these three assays is ACCESS (most equimolar) > OPUS > Stratus (least equimolar) may explain those data. An assay that detects both forms of cTnI equally well could be an advantage over a method that detects only one form of cTnI because the presence of any form of cTnI in serum is indicative of cardiac damage.

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Glutamic Acid Decarboxylase Antibodies in Screening for Autoimmune Diabetes: Influence of Comorbidity, Age, and Sex on Specificity and Threshold Values,

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Antibodies against the 65-kDa isoform of glutamic acid decarboxylase (GAD_{65}) can be applied as a predictive tool for childhood type-1 diabetes (1–6) and to facilitate the differential diagnosis of diabetes in adults (7–9). However, the sensitivity and specificity of GAD antibody screening have not been fully characterized, and the positive predictive value of screening varies from 20% to 70%, depending on the strategy applied and the population studied (2, 3, 5–7, 9–12). The current study aims to identify factors that may lead to false-positive results in GAD antibody screening.

Previously, it has been demonstrated that the GAD antibody frequency in mixed connective tissue disease and stiff-man syndrome is increased, although not all patients who suffer from these diseases and are positive

for GAD antibodies develop type-1 diabetes (13, 14). GAD is expressed in the islets of Langerhans, neuronal tissue, the ovaries, and the testes (15, 16). Similar to the above-mentioned examples, comorbidity involving these tissues may lead to GAD antibody formation but not to diabetes. Therefore, we compared the prevalence of GAD antibodies in patients with cystic fibrosis, epilepsy, Guillain-Barré syndrome, and premature ovarian failure to the prevalence in an unselected population of 1403 schoolchildren.

In addition, thresholds for positivity for GAD antibodies have generally been defined in children. These thresholds might not be applicable when testing for type-1 diabetes in adults. Therefore, we studied whether GAD antibody concentrations are correlated to age and sex, and whether adjustment of assay thresholds to include these variables may improve screening specificity.

The frequencies of positive results for GAD antibodies and the concentrations of GAD antibodies were established in a population of 1403 schoolchildren, ages 10–12 years, without chronic diseases (17). During a 10-year follow-up, two of these children developed type-1 diabetes [ascertainment >96% (18)].

The influence of comorbidity on GAD antibody concentrations and the frequencies of positive results were studied in four patient populations. The subjects included 394 patients who participated in the Dutch study of epilepsy in childhood (19, 20). These patients were eligible for the current study if the diagnosis was confirmed on the basis of electroencephalograms or therapy and sufficient serum for antibody analysis was available. Sera collected within 2 months after the presenting seizure (mean duration, 0.7 months; n = 228) and at the longest disease duration available of each patient (mean duration, 12.2 months; range, 2–50 months; n = 294) were analyzed separately. The diagnosis and development of diabetes during follow-up (5 years) were recorded from the medical records. Forty-three serum samples from 38 cystic fibrosis patients (collected in 1990–1992) were analyzed for GAD antibodies. In addition, we studied 30 patients with premature ovarian failure and 28 patients with Guillain-Barré syndrome (14 males; age range, 19–64 years). All patient sera were stored at −80 °C.

The influence of age and sex on GAD antibody concentrations and the frequencies of positive results were studied in 1287 individuals from the city of Zoetermeer, who participated in a study of cardiovascular risk factors. Sera were collected in 1976 and stored at −20 °C until testing. The population is described in detail elsewhere (21). The demographic data of the populations are shown in Table 1.

The study protocols were approved by the appropriate medical ethics committees according to the Helsinki Declaration. Informed consent was obtained from all participants or their parents.

Sera were tested for GAD antibodies by radiobinding assay (RBA) (13) or immunoprecipitation and sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the Triton X-100 fraction of [^{35}S]methionine-labeled fetal rat

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TI   EORTC receptor and biomarker study group report: A sandwich enzyme-linked
        immunosorbent **assay** for vascular endothelial growth factor in
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EORTC Receptor and Biomarker Study Group report: A sandwich enzyme-linked immunosorbent assay for vascular endothelial growth factor in blood and tumor tissue extracts

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ABSTRACT: A four-antibody sandwich enzyme-linked immunosorbent assay (ELISA) for vascular endothelial growth factor (VEGF) for application in blood (serum and plasma) and tumor tissue extracts was set up within the framework of the EORTC Receptor and Biomarker Study Group (RBSG). Polyclonal antibodies against $VEGF_{165}$ were raised in chickens and rabbits, and used in a previously described assay format. The assay was validated and characterized for use in serum, plasma and tumor tissue extracts. The resulting VEGF ELISA was found to be specific for $VEGF_{165}$ and $VEGF_{121}$, the main isoforms of VEGF. The assay showed good precision and parallelism in serial dilutions of samples. The assay was not susceptible to interference by heterophilic antibodies because avian antibodies (duck anti-chicken and chicken anti-VEGF) were used in the pre-analyte stage and mammalian antibodies (rabbit anti-VEGF and goat anti-rabbit) in the post-analyte stage. In conclusion, a sensitive, robust and specific VEGF ELISA has been developed. Research into the prognostic value of VEGF employing this assay is currently underway. (Int J Biol Markers, 2000; 15: 184-91)

Key w rds: Cancer, Vascular Endothelial Growth Factor (VEGF), Sandwich immunosorbent assay, Tumor tissue, Prognostic factors

INTRODUCTION

The formation of new vasculature, i.e. angiogenesis, is essential for the growth of solid tumors. This process is tightly regulated by a number of angiogenic and antiangiogenic substances (1, 2). Research has focused on the use of these factors as prognostic tumor markers or as targets for (antiangiogenic) therapy (3,4). Vascular endothelial growth factor (VEGF), one of the prominent angiogenic peptides, has been shown to be a potential prognostic marker in a variety of cancers (3, 5-9).

For the measurement of VEGF in tumor tissue or blood, several analytical difficulties exist: VEGF is a dimeric glycoprotein of which at least five isoforms exist due to alternative mRNA splicing, i.e. $VEGF_{121}$, $VEGF_{145}$, $VEGF_{165}$, $VEGF_{189}$ and $VEGF_{206}$ (for review, see 10). In tissue, $VEGF_{165}$ is the predominant isoform, whereas $VEGF_{121}$ and $VEGF_{165}$ are secreted into the circulation (10). Furthermore, related peptides have been described, i.e. placenta growth factor (PlGF), platelet-derived growth factor (PDGF), and VEGF-B, C, D and E (10,11), all of which could potentially interfere in immunological

assays for VEGF. For the assay of VEGF in blood, a major problem is the storage of VEGF in platelets. This leads to widely varying amounts of VEGF measured in plasma or serum. A correlation between the amount of platelets in blood and the concentration of VEGF in serum has been described, and the anticoagulant used to obtain plasma has an effect on the amount of VEGF recovered (12-14). Thus, to quantify VEGF concentrations in blood or tumor tissue, the sample preparation procedure should be well standardized and the VEGF assay should be analytically-chemically scrutinized.

Multicenter cancer studies in Europe are normally coordinated by the European Organisation for Research and Treatment of Cancer (EORTC). The EORTC Receptor and Biomarker Study Group (RBSG) was established to research and advise on common, or equivalent, methodologies for both steroid receptor and other biomarker assays, and to ensure that an appropriate quality assurance (QA) scheme is applied to all laboratories measuring biomarkers in material from patients entering EORTC-administered studies/trials. Our department, as the central QA laboratory of the RBSG, recently developed several

sensitive and specific assays for components of the plasminogen activator system (15,16) based on a four-antibody sandwich enzyme-linked immunosorbent assay (ELISA) technique that shows minimal interference by rheumatoid factor or heterophilic antibodies. These antibodies interfere with many ELISA techniques, in particular in blood and, to a lesser extent, in tumor tissue extracts, leading to erroneous results (17).

Here we describe an ELISA for VEGF based on a four-antibody concept and characterize the assay for application in both blood and tumor tissue extracts. The objective was to devise a sensitive and robust assay, specific for VEGF, insensitive to interfering substances, and widely applicable for research into the value of VEGF as a tumor marker within the RBSG.

MATERIAL AND METHODS

Equipment

Nunc Maxisorp flat-bottomed immunoplates were provided by Life Technologies (Breda, The Netherlands). The washing procedures were performed using a 96PW plate washer (SLT Lab Instruments GmbH, Salzburg, Austria). Optical densities (OD) were measured using an automated ELISA reader (Lab Systems, Oy, Helsinki, Finland) at 492 nm.

Reagents

Orthophenylenediamine (OPD) tablets (2 mg per tablet) were from Dako A/S (Glostrup, Denmark). Goat anti-rabbit IgG labeled with horseradish peroxidase (HRP) (#A-0545, Sigma Chemical Co, St Louis, MO, USA) was used as detecting antibody in our assays. Bovine serum albumin (BSA) (#A-7906) and sodium perborate capsules (#P-4922) were supplied by Sigma. All other reagents used were of analytical grade. The Quantikine VEGF ELISA kit was obtained from R&D Systems Inc. (Minneapolis, MD, USA).

The composition of the buffers was as follows: Coating buffer, 15 mmol/L Na_2CO_3 and 35 mmol/L $NaHCO_3$, pH 9.6. Phosphate buffered saline (PBS), 14 mmol/L NaCl, 2.7 mmol/L KCl, 1.5 mmol/L KH_2PO_2 and 8.1 mmol/L Na_2HPO_4, pH 7.4. Blocking buffer, 2% BSA in PBS. Washing buffer, 0.1% Tween-20 in PBS. Dilution buffer, 1% BSA in washing buffer. Color buffer, 0.05 M citrate-phosphate buffer containing 0.03% sodium perborate, pH 5.0, was obtained by dissolving one capsule of sodium perborate in 100 mL aqua dest. EORTC buffer (proposed by the EORTC (18), 20 mmol/L K_2HPO_4/KH_2PO_4, 1.5 mmol/L K_2EDTA, 3 mmol/L sodium azide, 10 mmol/L monothioglycerol, 10% [v/v] glycerol/water, pH 7.4. Substrate solution contained 4 mg OPD in 11 mL color buffer. High performance ELISA (HPE) buffer was provided by Research Diagnostics Inc. (Flanders, NJ, USA).

For immunization, recombinant human (rh)$VEGF_{165}$ was obtained from R&D Systems. For specificity tests placenta growth factor (PlGF) and nerve growth factor (NGF) were obtained from R&D Systems. Platelet-derived growth factor-B (PDGF-B) was obtained from Dr Hoppe (Würzburg, Germany), tumor-necrosis factor-α (TNFα) from Genentech Inc. (South San Francisco, CA), and transforming growth factor-α (TGFα) from PeproTech Inc. (Rocky Hill, NJ). Human growth hormone (hGH, Genotropin) was from Pharmacia (Uppsala, Sweden) and insulin-like growth factor-1 (IGF-1) from Peninsula (Belmont, CA).

Purification of VEGF

$VEGF_{165}$ for affinity-purification of antibodies was prepared from medium of baby hamster kidney (BHK) cells expressing rh$VEGF_{165}$, which were kindly provided by NovoNordisk (NovoNordisk A/S, Gentofte, Denmark). Cell medium was treated with ammonium sulphate, after which the resulting precipitate was dialyzed against 50 mM NaCl/10 mM Tris-HCl, pH 7.2. VEGF was subsequently partially purified with heparin sepharose beads (Pharmacia), after which the purified fraction was lyophilized using a SpeedVac vacuum concentrator (Savant, Holbrook, NY) and redissolved in 2.5 mL of 4 M guadinium-HCl with 30 µM dithiotreitol (DTT). Further purification was performed using SepPak C4 cartridges (Waters Corp., Milford, MA, USA). VEGF was finally purified applying high performance liquid chromatography (Waters) equipped with a Macrosphere RP 300 C8 5U HPLC column (Alltech, Breda, The Netherlands) 150x4.6 mm. A linear gradient of 100% solvent A (acetonitrile - 7.5%; isopropanol - 2.5%; trifluoroacetic acid (TFA) - 0.1%) up to 100% of solvent B (acetonitrile - 52.5%; isopropanol - 17.5%; TFA - 0.1%) after 40 min was followed by a 5 min isocratic run of solvent B at a flow of 1 mL/min. The operational column temperature was 37°C. Fractions were collected based on protein content (absorption at 280 nm) and analyzed for VEGF content using a commercially available VEGF ELISA kit (Quantikine, R&D Systems). The purity of the resulting VEGF preparation was analyzed by Western blot. A single band of 24 kDa was observed after staining with a commercially available biotinylated polyclonal anti-human VEGF antibody (R&D Systems) corresponding with the approximate size of monomeric $VEGF_{165}$. Under non-reducing conditions, a band of 45 kDa, i.e. the size of homodimeric $VEGF_{165}$, was found. This indicated the identity and purity of the sample used for subsequent column affinity chromatography of the polyclonal antibodies raised against VEGF.

Polyclonal antibodies

Antiserum to chicken IgY was raised in duck, whereas antibodies against $rhVEGF_{165}$ were raised in chickens and rabbits as described earlier (15). The blood samples from rabbits were collected in citrate tubes and the obtained plasma was stored at -20°C. Isolation of the IgY fraction from the chicken egg yolk was performed as described earlier (15). The duck IgY was obtained after chloroform and polyethyleneglycol-6000 precipitation essentially as described earlier (19). Isolates were stored at -20°C. All polyclonal anti-VEGF antibodies were purified by affinity chromatography on $VEGF_{165}$coated columns (AffiGel 15, Bio-Rad Laboratories, Hercules, CA) as described earlier (15, 16). All purified antibodies were diluted with glycerol (1:1) prior to storage and stored in aliquots at -20°C. Under these conditions the purified antibodies can be used for at least 12 months.

ELISA procedure

All incubation steps were separated by washing steps (4 times, 300 µL of washing buffer per well). The coating antibody was diluted in the coating buffer, whereas catching, tagging and detecting antibodies were diluted in dilution buffer. Antibodies were added in a total volume of 100 µL per well. For the assay of tumor tissue extracts, standards, cytosols and reference samples were diluted in dilution buffer, whereas in case of blood samples, standards, plasma, sera and reference samples were diluted in HPE buffer. The procedure was started by treating the microtiter plates with coating antibody solution (3.2 mg/L), i.e. duck anti-chicken IgY overnight at 4°C. Next, the plates were blocked with the blocking buffer (300 µL per well, 2 h at 37°C). The next step was the incubation with catching antibody solution (0.6 mg/L), i.e. chicken anti-VEGF (2 h at 37°C). Incubation with the standards, unknowns and reference samples took place overnight at 4°C. The incubation with tagging antibody (rabbit anti-VEGF, 0.75 mg/L) and the subsequent incubation with the detecting antibody (HRP-labeled goat anti-rabbit, dilution 20,000 times) were performed for 2 h at ambient temperature. The incubation with substrate solution was performed in darkness for 60 min at ambient temperature. The color reaction was stopped by the addition of 100 µL of 1 M H_2SO_4 per well. The optical density was measured at 492 nm within 30 min.

Preparation of cytosol, serum and plasma

The primary breast cancer biopsies were taken from our own tumor bank. Frozen tissues were pulverized with liquid nitrogen using a microdismembrator (Braun, Melsungen, Germany), suspended in EORTC buffer, and centrifuged at 800xg and 105,000xg at 4°C. High-speed supernatants (cytosols) were divided into aliquots and stored at -80°C until further analysis. Prior to the assay, cytosols were diluted 10 times in dilution buffer.

Sera and plasma (EDTA) samples were routine patient samples collected at the Department of Chemical Endocrinology and stored at –80°C until analysis. These samples were diluted five times in HPE buffer before use.

Statistical methods

All measurements were performed in duplicate. The standard curves were approximated by a four-parametric curve by a method of least squares. A linear regression procedure as described by Passing and Bablok (20, 21) was used to compare our assay with the R&D Systems Quantikine VEGF ELISA kit. Correlation coefficients were calculated according to Spearman. In this comparison difference plot analysis (22) was also applied.

RESULTS

Standard curves and sensitivities

The dose response curves of VEGF standards either diluted in HPE buffer for the assay of VEGF in blood or diluted in dilution buffer for the assay in tumor tissue extracts are shown in Figure 1A.

The analytical sensitivity, defined as the amount of VEGF giving a signal in the ELISA greater than two standard deviations above blank values, was 6 pg/mL for the assay in sera and plasma samples. For cytosols the analytical sensitivity was 5 pg/mL. The functional sensitivity, i.e. the amount of VEGF that could be measured with a precision of at least 20%, was approximately 12 pg/mL for the assay in blood and tumor tissue (inserts of Fig. 1B and 1C, respectively).

Precision and stability

The within-assay precision was assessed by plotting the variation in duplicate samples against the VEGF concentration. For serum samples (n=250) the variation differed from approximately 18% at 20 pg/mL to 7% at higher (>200 pg/mL) levels of VEGF (Fig. 1B). In cytosols (n=110) the within-assay precision ranged from 16% at 20 pg/mL to 6% at higher levels (Fig. 1C).

The between-assay precision was assessed by analyzing a reference serum sample in each assay. During a period of nine months no discernible changes in VEGF concentration (median 500 ng/mL, range 400-623) were observed in this sample. The inter-assay precision in the 36 assays performed in this period was 12.2 %.

Specificity

The assay was found to be specific for $VEGF_{165}$ and

$VEGF_{121}$. On a molar basis $VEGF_{121}$ cross-reacted 100%. Other substances, i.e. PlGF, NGF, PDGF-B, TNFα, TGFα, hGH and IGF-1, showed undetectable signals in the ELISA at concentrations up to 200 ng/mL (data not shown).

Recovery and parallelism

Recovery of $rhVEGF_{165}$ was studied by adding known quantities at two different concentrations to human sera (n=6) and cytosols (n=6). In the sera, which endogenously contained 96 to 201 pg/mL VEGF (median 129 pg/mL), recovery of 300 or 600 pg/mL added VEGF was 104 ± 11% (mean ± SD). In cytosols, which contained 105 to 294 pg/mL VEGF (median 180 pg/mL), a recovery of 96 ± 5% (mean ± SD)of added $rhVEGF_{165}$ at 240 or 480 pg/mL was found. In either sera or cytosols no difference in recovery was observed between the two concentrations of added VEGF.

Sera (n=7) and plasma (n=8) samples, diluted 3, 6 or 12 times in HPE buffer, exhibited excellent parallelism (Fig. 2A and 2B). Cytosol fractions (n=6) in four serial dilutions of 7.5 to 60 times in dilution buffer also exhibited excellent parallelism between dilutions (Fig. 2C).

Interfering substances

The robustness of the assay was checked by replacing the tagging antibody by a different (anti-urokinase plasminogen activator) antibody. Signals obtained by this assay format are indicative of aspecific binding of components of the assay, and of sensitivity of the assay for interfering substances like rheumatoid factor, human anti-mouse (or anti-mammalian, HAMA) or heterophilic antibodies which can cross-link pre- and post-analyte antibodies. Using the four-antibody assay with duck and chicken antibodies in the pre-analyte stage and rabbit and goat antibodies in the post-analyte stage, no signal was obtained in serum or plasma samples. However, replacement of the duck anti-chicken IgY by sheep anti-chicken IgG resulted in frequent (56%) false positives in 25 serum samples known to contain HAMAs.

Sera from pregnant women are known to contain binding proteins that completely block the binding of VEGF by monoclonal antibodies in an ELISA (23,24). In fact, in our assay almost 60% of maternal sera showed VEGF levels below the limit of detection. A median VEGF level of 0.04 ng/mL was found. This indicated that our polyclonal ELISA suffers from interference by a pregnancy-associated VEGF binding protein.

Comparison of the present assay with the Quantikine VEGF ELISA

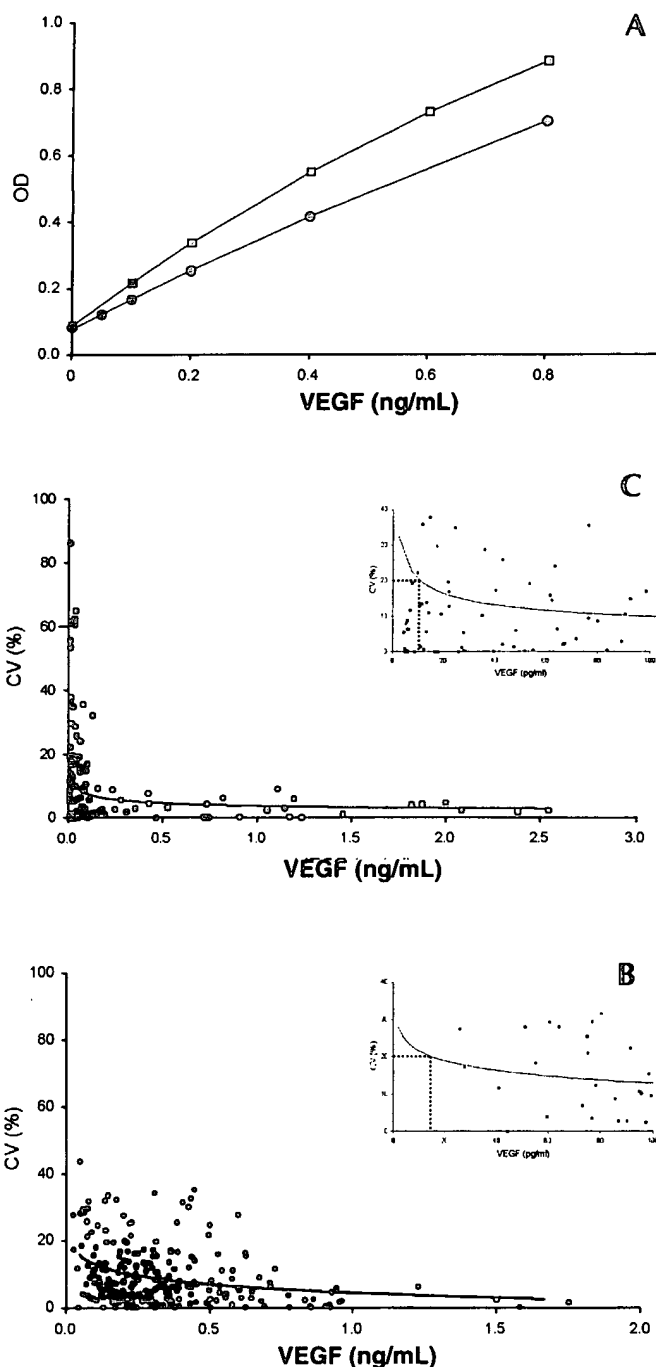Values obtained by the assay described here were

Fig. 1 - A) standard curves of VEGF diluted in dilution buffer for cytosol assay (■) or in HPE buffer for the assay of VEGF in blood (●). Precision profiles for VEGF assay in blood (B) or tissue (C) samples. Inserts show functional sensitivity as the amount of VEGF measurable at 20% variation. OD = optical density.

compared with values obtained by a commercially available VEGF ELISA (Quantikine, R&D Systems).

In serum samples (n=39) the median VEGF values obtained with the present method (range 0-1.65, median 0.45 ng/mL) were approximately 1.6 times higher than
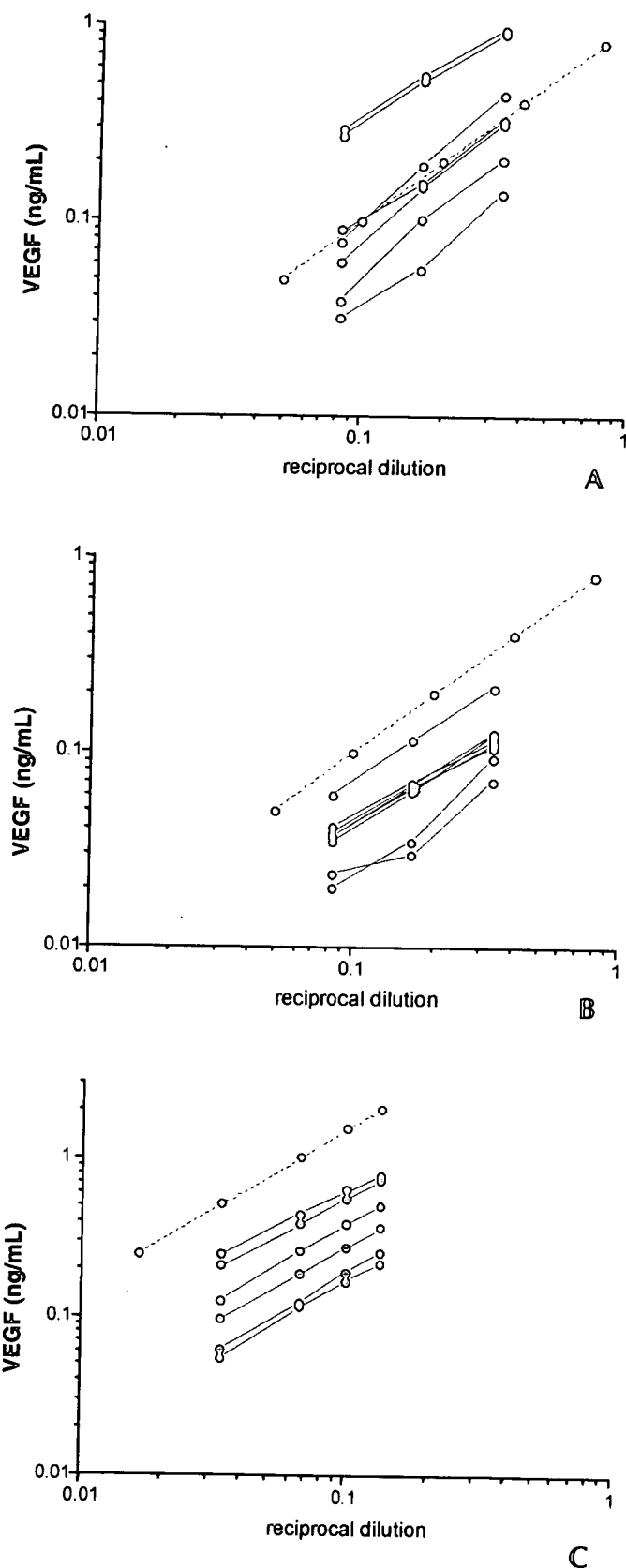
Fig. 2 - *Serial dilutions of (A) serum, (B) plasma and (C) cytosol samples. Serial dilutions of standards are shown as dotted lines.*

those obtained with the Quantikine VEGF assay (range: 0.05-1.14, median 0.28 ng/mL) (Fig. 3A). Regression analysis according to the method of Passing and Bablok (20, 21) gave the following equation: FS = 1.283*R&D + 0.06 (FS = Four-Span, our method; R&D = Quantikine VEGF ELISA). Spearman's correlation was 0.794 (p<0.001).

In plasma samples (n=45) the median VEGF values obtained with the present method (range 0-4.48, median 0.32 ng/mL) were twice those obtained with the Quantikine assay (range 0-2.82, median 0.16 ng/mL) (Fig. 3B). In both assays values obtained in plasma were lower than those obtained in serum samples. Regression analysis gave the following equation: FS = 1.876*R&D + 0.013. Spearman's correlation was 0.854 (p<0.001).

In cytosols (n=40) the median values obtained with the present method (range 0-41.96, median 0.52 ng/mL) were three times higher than those obtained with the Quantikine assay (range 0-8.84, median 0.18 ng/mL) (Fig. 3C). Regression analysis gave the following equation: FS = 2.037*R&D + 0.100. Spearman's correlation was 0.934 (p<0.001).

When difference plots were applied (Fig. 3, right panels), it could be confirmed that the present assay leads to higher values than the R&D Systems VEGF ELISA kit. No trend was observed.

DISCUSSION

In this article we describe a four-antibody sandwich ELISA for VEGF using polyclonal antibodies. The ELISA is specific for $VEGF_{121}$ and $VEGF_{165}$ with a functional sensitivity of 12 to 14 pg/mL in sera and cytosols, respectively. The acquired data are comparable with those obtained with a commercial ELISA for VEGF (R&D Systems), although higher values are obtained with our assay. The described assay exhibits no interference by HAMAs.

The fact that we found higher values for VEGF concentrations in serum, plasma and cytosols than the Quantikine VEGF ELISA might be attributable to a differential sensitivity for the different isoforms of VEGF. Our assay shows 100% specificity for both $VEGF_{165}$ and $VEGF_{121}$, whereas the specificity of the Quantikine ELISA is not described by the manufacturer. Since a monoclonal antibody is used in the commercial assay, this assay might be less sensitive for $VEGF_{121}$ than our assay, which would explain the higher values obtained by us. We did exclude cross-reaction of several related growth factors in our assay. However, the occurrence of homo- and het-erodimers of several of the isoforms of VEGF, with unknown cross-reactions in the tested assays, precludes a complete explanation for the established differences.

The functional sensitivity of the assay, 12 pg/mL, compares favorably with fluorimetric (25) or chemilumi-
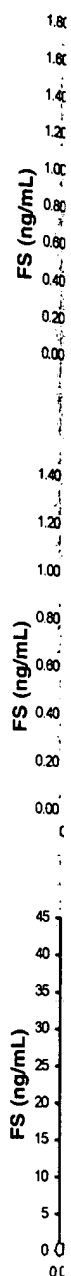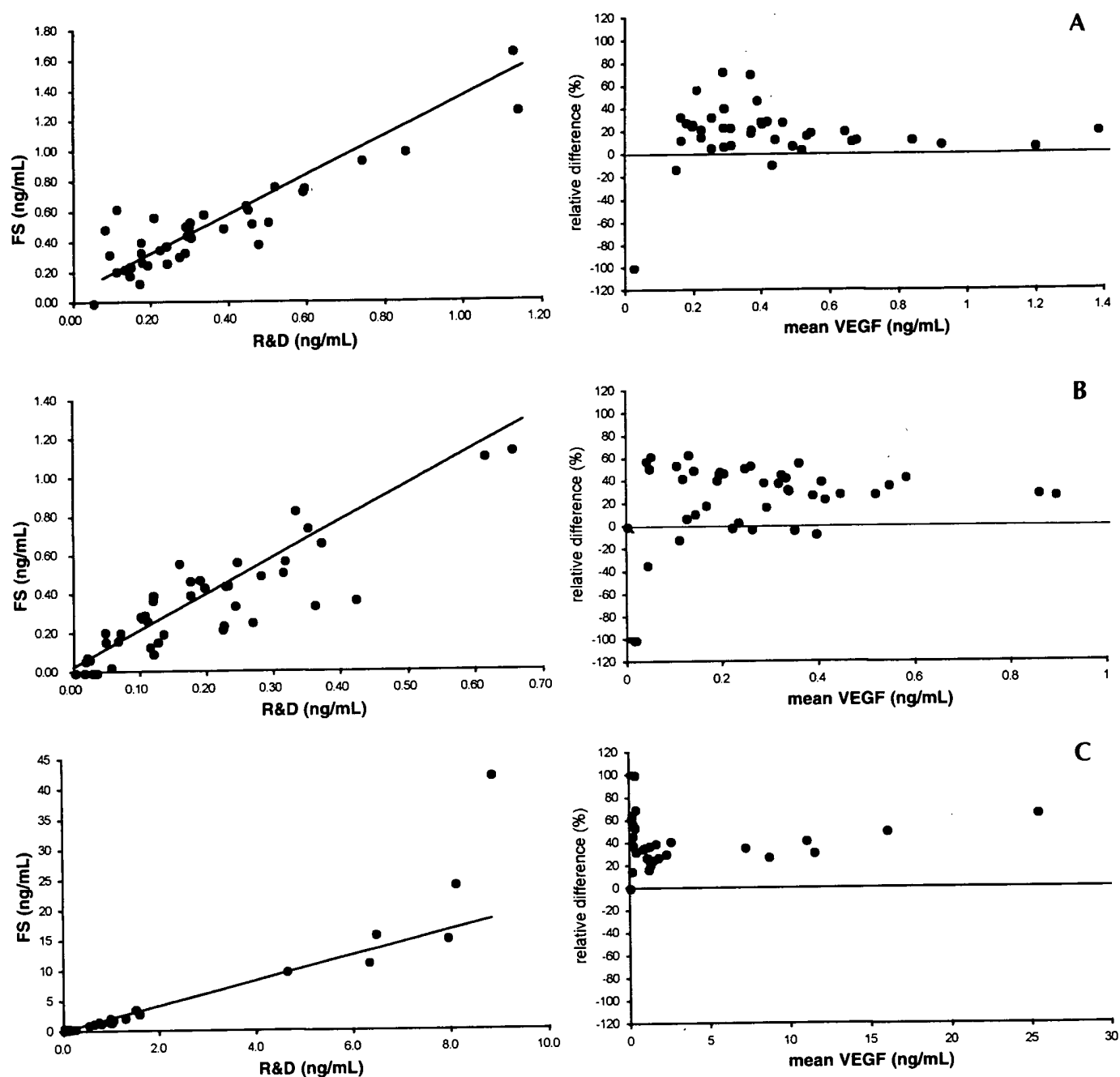
Fig. 3 - *Relationship (left) and agreement (right) between the results obtained with our assay (FS, Four-Span) and the Quantikine VEGF ELISA kit (R&D) in (A) serum, (B) plasma and (C) cytosol samples. Regression is performed according to Passing and Bablok (19, 20).*

nescent (26, 27) ELISAs for VEGF described earlier. However, in contrast to assays employing signal enhancing procedures, the colorimetric assay described here can be performed with equipment and materials available to most laboratories. The relatively high sensitivity can partially be explained by the use of avian antibodies, which usually exhibit greater affinity for human antigens than mammalian antibodies. Furthermore, the use of these antibodies results in an assay that is less susceptible to in-

terfering substances (see below). Therefore, samples do not have to be diluted to minimize matrix effects as much as when mammalian antibodies are used, resulting in greater functional sensitivity. This is of particular interest for the measurement of VEGF in plasma, which contains less VEGF than serum. Extensive dilution of plasma samples would lead to a considerable number of samples being below the limit of detection.

The finding that plasma samples contain less VEGF

than serum samples is attributable to the reported substantial storage of VEGF in platelets (13). This platelet-bound VEGF is released during clotting to obtain serum, whereas during the preparation of plasma samples platelet-bound VEGF is not released. The significance, as a prognostic marker, of platelet-bound or non-bound VEGF is the subject of research (14, 28). From an analytical point of view, however, it should be taken into account that determination of VEGF in serum or plasma would lead to results that need to be interpreted differently.

The ELISA for VEGF described here is similar to the assays for components of the plasminogen activator system we have described previously (15, 16). The four-antibody sandwich ELISA format is multiapplicable, as the exchange of the specific antibodies by antibodies against VEGF leads to a sensitive and specific assay without extensive modifications in tumor cytosols. However, for the assay of VEGF in serum or plasma samples, additional modifications are necessary. Initially, serial dilutions of blood samples exhibited poor parallelism when diluted in dilution buffer. Thus, the calculated amount of VEGF present in undiluted samples changed when the sample was diluted. This problem was overcome by using the commercially available HPE buffer from Research Diagnostics Inc. (Flanders, NJ). Dilution of samples and standards in this buffer resulted in excellent parallelism between serial dilutions.

Another frequent problem encountered in ELISA assays is the susceptibility of the assay to interfering substances such as rheumatoid factor, human anti-mouse antibodies (HAMAs) or heterophilic antibodies (17, 29-31). These antibodies appear frequently in patient samples and lead to false positive values by aspecifically cross-linking pre- and post-analyte antibodies. As shown here, the use of avian antibodies in the pre-analyte stage and mammalian antibodies in the post-analyte stage (31) results in an assay without false positive results due to HAMA or rheumatoid factor interference.

In pregnancy, VEGF in maternal sera has been described as not detectable by the Quantikine ELISA (23) for VEGF due to binding of an unknown protein of approximately 400-700 kDa (24). When assayed by a competitive radioimmunoassay (RIA), VEGF could be readily determined (23,32). As we use polyclonal antibodies, in contrast to the monoclonal antibody used in the Quantikine VEGF ELISA, we hypothesized that our assay might not, or to a lesser extent, be influenced by this binding protein. Therefore, we assessed the amount of VEGF in sera obtained from pregnant women. In approximately half of these samples VEGF was detectable using our assay. Thus, it seems that our polyclonal assay is less susceptible to interference by pregnancy-associated VEGF binding proteins than ELISAs in which a monoclonal antibody is used. However, considering the reported elevated VEGF levels in pregnancy assessed by competitive immunoassays (32), it should be concluded that our as-

say does suffer from partial interference and is less suited for application in pregnancy than RIA.

The measurement of vascular endothelial growth factor (VEGF), a prominent angiogenic peptide, in blood or tumor tissue has been shown to be a potential prognostic marker in a variety of cancers (3, 5-9). Within the framework of the Receptor and Biomarker Study Group (RBSG) of the European Organisation for Research and Treatment of Cancer (EORTC) we developed a robust, sensitive and specific assay for VEGF based on an earlier developed and characterized four-antibody sandwich ELISA method (15, 16). The ELISA described here is available to the laboratories involved in the validation of assays for biomarkers in malignancies through the RBSG. The assay is highly specific for $VEGF_{121}$ and $VEGF_{165}$. These isoforms of VEGF can be secreted into the bloodstream. Furthermore, a significant fraction of $VEGF_{165}$ is bound to the extracellular matrix, making it a predominant tissue isoform (10). Thus, the assay is particularly suited for measuring tumor-derived VEGF. Several studies on the potential predictive or prognostic value of VEGF are being undertaken by laboratories associated with the RBSG.

ACKNOWLEDGEMENTS

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L4   ANSWER 3 OF 8 CAPLUS COPYRIGHT 2003 ACS
AB   A four-antibody sandwich ELISA for vascular endothelial growth factor
      (VEGF) for application in blood (serum and plasma) and tumor tissue exts.
      was set up within the framework of the EORTC Receptor and Biomarker Study
      Group (RBSG). Polyclonal antibodies against VEGF165 were raised in
      chickens and rabbits, and used in a previously described **assay**
      format. The **assay** was validated and characterized for use in
      serum, plasma and tumor tissue exts. The resulting VEGF ELISA was found
      to be specific for VEGF165 and VEGF121, the main **isoforms** of
      VEGF. The **assay** showed good precision and parallelism in serial
      dilns. of samples. The **assay** was not susceptible to
      interference by heterophilic antibodies because avian antibodies (duck
      anti-chicken and chicken anti-VEGF) were used in the pre-**analyte**
      stage and mammalian antibodies (rabbit anti-VEGF and goat anti-rabbit) in
      the post-**analyte** stage. In conclusion, a sensitive, robust and
      specific VEGF ELISA has been developed. Research into the prognostic
      value of VEGF employing this **assay** is currently underway.

=>

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AB   The immunogenicity of a multiple antigenic peptide construct consisting
of

      four copies of the synthetic 21-mer peptide DANFDSIRVDAVDNVDADLLQ was
      measured. The composition of this peptide was derived from a sequence in
      the N-terminal region of mutans streptococcal glucosyltransferases (GTFs)
      containing an aspartic acid implicated in catalysis. The peptide (CAT)
      construct was synthesized as a tetramer on a lysine backbone and
      subcutaneously injected into Sprague-Dawley rats for polyclonal antibody
      formation or intraperitoneally injected into BALB/c mice, and then spleen
      cell fused with Sp2/OAg14 murine myeloma cells for monoclonal antibody
      formation. The resulting rat antisera and mouse monoclonal antibodies
      reacted with CAT and with native GTF isozymes from Streptococcus sobrinus
      and Streptococcus mutans (in enzyme-linked immunosorbent **assay**
      and Western blot (immunoblot) analyses). Functional inhibition of the
      water-insoluble glucan synthetic activity of S. sobrinus GTF-I was
      demonstrated with an immunoglobulin M anti-CAT monoclonal antibody (gt
      80% inhibited) and with rat sera (approximately 17% inhibited). The
      monoclonal antibody preparation also modestly inhibited the water-soluble
      glucan synthetic activity of an S. mutans GTF mixture. These results
      suggest that the CAT peptide contains B-cell epitopes that are similar to
      those of intact mutans streptococcal GTFs and has the potential to elicit
      antibody that can inhibit GTF function. Thus, sequences within this
      peptide construct may have value for inclusion in a synthetic dental
      caries vaccine.

7/25/03

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Sent:	Friday, July 18, 2003 11:45 AM
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Subject:	reference - 09/824,587

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Thank You
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Immunological Characteristics of a Synthetic Peptide Associated with a Catalytic Domain of Mutans Streptococcal Glucosyltransferase

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The immunogenicity of a multiple antigenic peptide construct consisting of four copies of the synthetic 21-mer peptide DANFDSIRVDAVDNVDADLLQ was measured. The composition of this peptide was derived from a sequence in the N-terminal region of mutans streptococcal glucosyltransferases (GTFs) containing an aspartic acid implicated in catalysis. The peptide (CAT) construct was synthesized as a tetramer on a lysine backbone and subcutaneously injected into Sprague-Dawley rats for polyclonal antibody formation or intraperitoneally injected into BALB/c mice, and then spleen cell fused with Sp2/0Ag14 murine myeloma cells for monoclonal antibody formation. The resulting rat antisera and mouse monoclonal antibodies reacted with CAT and with native GTF isozymes from Streptococcus sobrinus and Streptococcus mutans (in enzyme-linked immunosorbent assay and Western blot [immunoblot] analyses). Functional inhibition of the water-insoluble glucan synthetic activity of S. sobrinus GTF-I was demonstrated with an immunoglobulin M anti-CAT monoclonal antibody (>80% inhibited) and with rat sera (approximately 17% inhibited). The monoclonal antibody preparation also modestly inhibited the water-soluble glucan synthetic activity of an S. mutans GTF mixture. These results suggest that the CAT peptide contains B-cell epitopes that are similar to those of intact mutans streptococcal GTFs and has the potential to elicit antibody that can inhibit GTF function. Thus, sequences within this peptide construct may have value for inclusion in a synthetic dental caries vaccine.

Dental caries in humans is associated primarily with the acidogenic and aciduric properties of the mutans streptococci (13). The colonization and accumulation of these cariogenic oral streptococci on the tooth surface have been associated with the ability of mutans streptococci to synthesize extracellular glucan from sucrose (9). Mutans streptococcal glucans are synthesized by glucosyltransferase enzymes (GTFs). The glucan-mediated accumulation of cariogenic mutans streptococci in dental plaque occurs through its interaction with cell-associated glucan-binding proteins (16). Immunologic interference with (25, 27) or genetic alteration of (14) glucan synthetic mechanisms has been associated with a reduction in mutans streptococcal cariogenicity. Thus, the catalytic activity of GTFs can be viewed as a virulence trait of mutans streptococci.

We have begun to explore the potential for development of subunit dental caries vaccines based on epitopes derived from GTF. Epitopes associated with enzyme function would theoretically be primary targets, provided that the relevant sequence(s) was located in molecular areas that are accessible to antibody. The location of a catalytic domain in the N-terminal third of GTF has been suggested by Mooser and coworkers (19, 20) on the basis of identification of an aspartic acid in this region that was found to be radiolabelled with glucose after pepsin digestion of GTF that had been rapidly denatured after incubation with radiolabelled sucrose. The aim of the present study was to synthesize and test a peptide whose sequence was derived from the highly conserved region containing this putative catalytic site within the GTF molecule. The synthetic design included four copies of this peptide (CAT) on a lysine backbone (26); this design may potentially enhance immunogenicity while avoiding irrelevant responses that result from

the use of carrier proteins. The characteristics of polyclonal antibodies (rat) and monoclonal antibodies (MAbs; mouse) to the CAT construct were then evaluated with respect to reactivity with Streptococcus sobrinus and Streptococcus mutans GTF and by functional inhibition of GTF activity.

MATERIALS AND METHODS

Antigens. (i) CAT synthetic peptide. We selected for synthesis a 21-mer peptide, DANFDSIRVDAVDNVDADLLQ, that had complete homology with the derived sequences of residues 444 to 464 of Streptococcus downei GTF-I (22) and residues 442 to 462 of S. mutans GTF-B (25) (Table 1). The sequences of these two mutans streptococcal GTFs contain the aspartic acid (residues 453 [S. downei] and 451 [S. mutans GTF-B]) that has been suggested by Mooser et al. (19) to be the residue to which glucose is covalently bound in the active site. This sequence is highly conserved among mutans streptococcal GTFs (1, 6, 9, 10, 14) (Table 1). The peptide was synthesized (Applied Diagnostics, Foster City, Calif.) by using the stepwise solid-phase method of Merrifield (17) on a core matrix of three lysines to yield a multiple antigenic peptide macromolecule with four identical 21-mer peptides per molecule, after the method of Tam (26). Purity (>90%) was assessed by high-performance liquid chromatography, amino acid analysis, and molecular weight determination by mass spectrometry. This multiple antigenic peptide construct, referred to as CAT, was used for immunization and antibody analyses.

(ii) GTFs. GTFs from S. sobrinus 6715 and S. mutans SJ were obtained as described previously (24). Briefly, after bacterial growth in glucose-containing defined media, enzymes were isolated by chr matography on Sephadex G-100 (Pharmacia Fine Chemicals), with 3 M guanidine HCl used as the eluting solvent. These GTF-rich pools were then subjected t fast pr tein liquid chromatography n Superose 6 (Pharma-

TABLE 1. Comparison of the synthetic peptide sequence with deduced sequences of GTFs from oral streptococci containing a putative active-site aspartic acid

Protein	Streptococcal species (reference)	Deduced sequence[a]	% Homology with synthetic peptide
		⇩	
GTF-I	S. downei (8, 22)	444 DANFDSIRVDAVDNVDADLLQ 464	100
	S. sobrinus (1)	438 ...........E......Q.. 458	91
	S. salivarius (7)	464 .....G...........M.. 483	91
GTF-S	S. downei (8)	428 .....GV........N..... 448	86
GTF-B	S. mutans (23)	442 462	100
GTF-C	S. mutans (10, 29)	487 507	100
GTF-D	S. mutans (11, 12)	456 E....GV........N..... 476	81
CAT synthetic peptide		DANFDSIRVDAVDNVDADLLQ	

[a] ⇩, indicates the aspartic acid putatively associated with the catalytic activity of GTF by Mooser and coworkers (19, 20).

cia), with 6 M guanidine used for elution. The gel filtration step removes non-GTF and other glucan-binding proteins from GTF preparations of S. mutans and S. sobrinus, as evidenced by the fact that the protein bands observed after sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were all associated with enzymatic activity after incubation of duplicate gels in sucrose. The S. mutans GTF preparation taken to this level of enrichment synthesized more than 95% of the water-soluble glucan by both tube and filter assays (27) and thus was analogous to the gtfD gene product of S. mutans GS5 (12, 13). This preparation was designated GTF_{Sm} and used for injection and enzyme-linked immunosorbent assay (ELISA).

S. sobrinus GTF preparations obtained after gel filtration on Superose 6 contained a mixture of GTF-I (water-insoluble glucan product), GTF-Sd (primer-dependent water-soluble glucan product), and GTF-Si (primer-independent water-soluble glucan product) (15, 16). This preparation was designated GTF_{Ss} and used for injection and ELISA.

S. sobrinus GTF-I and GTF-Sd, used for measuring enzyme inhibition by rat antisera, were obtained by anion-exchange chromatography of the Superose 6 pool on MONO-Q (Pharmacia) as described previously (24, 28). Virtually all of the glucan product of GTF-I synthesis was water insoluble when tested by either tube or filter assays (conditions reported in reference 24). Glucan synthesis by GTF-I was enhanced threefold in the presence of dextran T10 primer (Pharmacia). The quantitatively predominant GTF-Sd (16) synthesized a water-soluble product from sucrose in tube assays, which was enhanced sixfold in the presence of dextran primer. In filter assays, depending on the enzyme-substrate concentration, 8 to 30% of the glucan product of GTF-Sd synthesis was retained by the filter. Thus, for the purposes of the inhibition assay (see Tables 3 and 4), the enzyme activities of GTF-Sd were described empirically as water soluble (not retained by the filter) and water insoluble (retained by the filter).

Immunization. Sprague-Dawley strain CD 65-day-old male rats (Charles River Laboratories) were used for injection. Rats were injected subcutaneously in the flanks and back with (i) 50 µg of the CAT construct (n = 3), (ii) 25 µg of S. sobrinus 6715 GTF_{Ss} (n = 2), or (iii) buffer alone (sham immunization; n = 3). The initial injection included complete Freund adjuvant (Difco); three subsequent injections included incomplete Freund adjuvant. The additional injections were given at approximately 3-week intervals. Animals were bled pri r to injection and biweekly thr ughout the injecti n period. Rats were exsanguinated 88 days after the first injecti n (12 days after the final injection), and serum was prepared.

An additional 21 Sprague-Dawley strain CD rats were injected under a separate protocol. Two immunizations were given at 25 and 35 days of age. Rats were injected subcutaneously in the salivary gland region with either 10 µg of the CAT synthetic peptide construct (n = 7), 15 µg of GTF_{Ss} (n = 7), or buffer alone (n = 7). The initial injection included complete Freund adjuvant, and the second injection included incomplete Freund adjuvant. Animals were bled 10 days after the second injection, and serum was prepared.

MAb to the CAT peptide (immunoglobulin M [IgM] MAb_{CAT}) was prepared by intraperitoneal injection of male BALB/c mice with the CAT construct, which was bound to nitrocellulose. Other mice were injected intraperitoneally with mixtures of culture supernatants of laboratory strains of S. sobrinus and S. mutans (SB105 and SB1) that had been precipitated with ammonium sulfate at 50% saturation (IgG MAb_{GTF}). A third group of mice were injected with GTF (and other glucan-binding proteins) adsorbed onto Sephadex G-50 from 50% ammonium sulfate-precipitated S. sobrinus SB105 culture supernatant (IgM MAb_{GTF}). Spleen cells from mice with elevated serum antibody to the respective antigens were fused with Sp2/0Ag14 murine myeloma cells (ICN Biomedicals, Inc., Costa Mesa, Calif.). Hybridomas producing antibodies to GTF or CAT were detected in an ELISA using the CAT peptide construct or purified S. sobrinus GTF. Positive hybridomas were cloned by limiting dilution. MAbs 4721.2 (IgM MAb_{CAT}) and 4631.2 (IgM MAb_{GTF}) were prepared with concanavalin A (30) from clones expanded in hollow fibers (Tecmouse; Integra Biosciences, Inc., Woburn, Mass.). The IgG MAb 4688.1 (IgG MAb_{GTF}) was prepared from mouse ascites by protein A affinity chromatography.

ELISA. Blood was taken from the retroorbital sinus of each rat. Serum, obtained from the coagulated specimen after centrifugation, was sto red frozen at −20°C until use. Rat sera and mouse MAb reagents were tested for antibody activity by a previously described biotin-avidin, alkaline phosphatase ELISA method (25). Polystyrene microtiter plates (Flow Laboratories, McLean, Va.) were coated with either 1 µg of poly-DL-alanine-poly-L-lysine (approximate molecular mass, 153 kDa; Sigma Chemical Co., St. Louis, Mo.) per ml, 0.5 µg of the CAT peptide per ml, 0.5 µg of S. sobrinus 6715 GTF_{Ss} (Superose 6 pool containing GTF-I, GTF-Sd, and GTF-Si), or S. mutans SJ GTF_{Sm} (Superose 6 pool containing GTF-S activity) (24). Antibody activity was then measured by incubation with 1:50 to 1:10^6 dilutions of sera. Plates were then developed for IgG or IgM antibody with the appropriate biotinylated affinity-purified goat anti-rat or anti-m use gamma-chain or mu-chain reagents (Zymed Laboratories, South San Francisco, Calif.) and then with streptavidin-alkaline phosphatase (Zymed) and p-nitrophenylphosphate, in that order. Reactivity was then recorded as absorbance (A_{405}) with

a microplate reader (Biotek Instruments, Winooski, Vt.). Each immure serum or MAb reagent was assayed simultaneously with either the respective preimmune serum or an irrelevant MAb f the appropriate isotype. These preimmune serum or irrelevant MAb control absorbances were then subtracted from the values for similarly diluted immune sera. The end point of the assay was taken as the dilution of the subtracted immune serum absorbance that was at least three standard deviations above that of the reagent background.

Antibody inhibition of glucan synthesis. Rat sera and MAb mouse reagents were evaluated for their ability to inhibit glucan synthesis of *S. sobrinus* GTF$_S$, *S. sobrinus* GTF-I and GTF-Sd, or *S. mutans* GTF by a filter assay. Ten to 20-μl volumes of sera (diluted from 1:10 to 1:8,000 with 0.02 M sodium phosphate-buffered saline and 0.02% sodium azide [PBSA; pH 6.5]) from rats injected four times with antigen, IgG or IgM MAb (tested at concentrations ranging from 15 to 370 μg/ml), or mouse IgG MAb (MOPC 21; Sigma) or IgM MAb (TEPC 183; Sigma) were preincubated with the respective GTF for 1 h at 37°C in a total volume of 0.04 ml of PBSA. Then, 1.7 mg of sucrose and 44 nCi of [^{14}C-glucose]sucrose (approximately 100,000 cpm) were added in 0.2 ml of PBSA in the presence of 37 μg of dextran T10 (Pharmacia). Incubation proceeded for 18 h at 37°C, after which water-insoluble glucan was collected on Whatman GF/F glass fiber filters, the filters were washed with PBSA, and radioactivity was determined as described previously (28). Essentially, all of the glucan formed by *S. sobrinus* GTF-I was captured on the filter. Under the conditions of the assay, approximately 70 and 95% of the glucan formed by *S. sobrinus* GTF-Sd and *S. mutans* GTF, respectively, could be found in the filtrate (referred to as water-soluble glucan). Sera from rats injected twice were tested with *S. sobrinus* or *S. mutans* GTF in a slightly modified assay. The serum (1 μl) and the enzyme were combined in a final volume of 100 μl in PBSA and incubated for 2 h at 37°C. Thereafter, 100 μl of PBSA containing 0.85 mg of sucrose and 22 nCi of [^{14}C-glucose]sucrose (approximately 50,000 cpm) was added and incubated for 2 h at 37°C. Water-insoluble glucan was collected on Whatman GF/F glass fiber filters, the filters were washed with PBSA, and radioactivity was determined. Water-soluble glucan in the filtrate was precipitated with 70% ethanol after the addition of 4 mg of carrier dextran T10 and centrifuged, and radioactivity was determined (28).

Inhibition with IgM monomers. In other experiments, IgM MAb monomeric subunits were tested for their ability to inhibit the synthetic capacity of *S. sobrinus* GTF-I. Subunits were prepared from the control TEPC 183 IgM MAb and the IgM MAb$_{CAT}$ after the method of Miller and Metzger (18). Approximately 200 μg of IgM in 0.2 M Tris-NaCl (pH 8.0) was incubated with 0.05 M cysteine for 40 min at 25°C. The reduced subunits were then alkylated with a 10% molar excess of iodoacetamide in 0.2 M Tris-NaCl (pH 7.3) for 15 min at 25°C. Th. reduced and alkylated subunits were then dialyzed extensively at 25°C in 0.2 M Tris-NaCl (pH 8.5). Under these conditions, essentially all of the pentameric IgM was converted to the monomer as indicated by the elution positions of dialyzed preparations after chromatography on Superose 6 (Pharmacia). Monomeric IgM fractions were then pooled and used in the inhibition assays as described above.

Competitive inhibition with CAT construct. In some experiments, the CAT construct was preincubated with polyclonal rat anti-GTF sera or anti-CAT MAb reagents t assess the ability of the peptide to remove antibody-mediated inhibitory activity. In these experiments, immune reagents were diluted initially to give approximately 50% inhibiti n of *S. sobrinus*

TABLE 2. Antibody activity of polyclonal antibodies and MAbs in an ELISA

Reagent	Geometric mean reciprocal endpoint dilution[b] with ELISA coating antigen of:		
	S. sobrinus GTF[c]	S. mutans GTF[d]	CAT
IgM MAb$_{CAT}$	3.2×10^3	3.2×10^3	2.5×10^3
IgG MAb$_{GTF}$	5.1×10^4	2.5×10^4	$<10^2$
IgM MAb$_{GTF}$	3.2×10^3	3.2×10^3	$<10^2$
Polyclonal rat IgG anti-CAT	3.9×10^3	1.0×10^3	2.0×10^5
Polyclonal rat IgG anti-GTF$_S$	5.1×10^6	3.6×10^4	$<10^2$
Polyclonal sham rat IgG sera	$<10^2$	$<10^2$	$<10^2$

[a] Immunogens included CAT (IgM MAb$_{CAT}$; polyclonal rat anti-CAT). culture supernatants of *S. sobrinus* and *S. mutans* that had been precipitated with (NH$_4$)$_2$SO$_4$ (IgG MAb$_{GTF}$), *S. sobrinus* GTF adsorbed onto Sephadex G-50 from culture supernatants of *S. sobrinus* precipitated with (NH$_4$)$_2$SO$_4$ (IgM MAb$_{GTF}$), and a Superose pool containing a mixture of *S. sobrinus* GTF isozymes (polyclonal rat anti-GTF$_S$).

[b] The geometric mean reciprocal endpoint dilution of triplicate assays is given for each MAb or set of polyclonal rat sera ($n = 2$ to 3) after subtraction of corresponding preimmune or irrelevant MAb absorbances. The endpoint of the assay was taken as the dilution of the subtracted immune absorbance that was at least three standard deviations above that of the reagent background.

[c] Superose 6 pool of *S. sobrinus* GTF containing GTF-I, GTF-Sd, and GTF-Si (GTF$_S$).

[d] Superose 6 pool of *S. mutans* GTF.

GTF-I activity. CAT peptide, in a thousandfold range of concentrations, was then preincubated with immune and control reagents for 0.5 h at 37°C. After incubation of peptide, the assay for inhibition was conducted as described above.

RESULTS

Immunogenicity of the CAT synthetic peptide. (i) ELISA. IgG antibody to the CAT construct appeared in all rats after two subcutaneous injections of CAT (geometric mean = 2.0 × 10⁵) (Table 2) and continued to rise thereafter (not shown). GTF from *S. sobrinus* (GTF$_S$) elicited a serum IgG antibody response reactive with the homologous antigen at reciprocal dilutions greater than 10⁵ and at dilutions greater than 10⁴ with *S. mutans* GTF (Table 2). Sera from rats injected with CAT also reacted with both intact *S. sobrinus* and *S. mutans* GTF antigens, albeit at much lower dilutions than the anti-GTF sera, suggesting that a common epitope may exist on CAT and intact GTF.

Mouse MAbs to the CAT construct or to GTF-containing preparations were also tested for activity to these antigens in an ELISA (Table 2). Of these MAbs, only the IgM anti-CAT MAb (IgM MAb$_{CAT}$) showed a significant reaction with the CAT peptide. Each MAb preparation reacted with both *S. sobrinus* and *S. mutans* GTFs. The reactivity of the anti-CAT clone (IgM MAb$_{CAT}$) with GTF at reciprocal dilutions greater than 10⁵ confirmed that common epitopes exist on CAT and native GTF.

(ii) Western blot (immunoblot) analysis of rat polyclonal sera. The specific reactivity of IgG antibody in the sera of rats injected with CAT with *S. sobrinus* GTF-I was also demonstrated in Western blot analyses (Fig. 1). IgG antibody in rat antisera t *S. sobrinus* (lane 1) and *S. mutans* GTF (lane 2) reacted with *S. sobrinus* GTF-I. Most importantly, IgG antibody in sera fr m rats injected with CAT also reacted with *S. sobrinus* GTF-I (lane 3). No reaction was seen with serum from a control rat injected with adjuvant alone (lane 4).

◄ 200

◄ 116
◄ 97

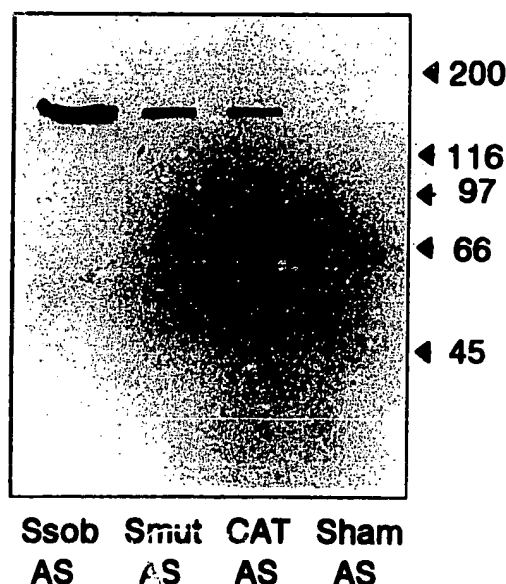◄ 66

◄ 45

Ssob Smut CAT Sham
AS   AS   AS   AS

FIG. 1. Western blot profiles of *S. sobrinus* GTF-I versus polyclonal rat sera to CAT and GTFs. *S. sobrinus* GTF-I was subjected to SDS-PAGE, transferred to nitrocellulose, exposed to polyclonal rat antisera (AS) to *S. sobrinus* GTF (Ssob AS; tested at a 1:500 dilution [lane 1]), *S. mutans* GTF (Smut AS; tested at a 1:500 dilution [lane 2]), CAT (CAT AS; tested at a 1:30 dilution [lane 3]), or buffer-adjuvant (Sham AS; tested at a 1:30 dilution [lane 4]), and subsequently developed with gamma chain-specific reagents as described in Materials and Methods. Migration positions of biotinylated molecular mass standards (kilodaltons) are indicated to the right of the figure.

Inhibition of GTF functional activity by antibody to CAT.

Sera from a group of rats, injected twice with CAT, were tested for their ability to inhibit the formation of water-soluble or -insoluble glucan from [^{14}C-glucose]sucrose by *S. sobrinus*

TABLE 3. Inhibition of *S. sobrinus* GTF$_{Ss}$-mediated glucan formation by rat polyclonal antisera to CAT or GTF$_{Ss}$

Antigen injected[a]	% Inhibition of WIG (mean ± SE)[c]	P[c]	% Inhibition of WSG (mean ± SE)	P
CAT (n = 7)	17.0 ± 4.6	<0.01	10.0 ± 3.2	<0.05
S. sobrinus GTF$_{Ss}$ (n = 6)	49.6 ± 2.6	<0.001	14.5 ± 2.8	<0.002

[a] Female Sprague-Dawley rats were injected twice 1 week apart with the indicated antigen incorporated in complete Freund adjuvant (first injection) or incomplete Freund adjuvant (second injection). Serum was collected 1 week after the second injection and tested at a level of 1 µl per assay.
[b] Incorporation of [^{14}C]glucose into water-insoluble glucan (WIG) or water-soluble glucan (WSG), synthesized from [^{14}C-glucose]sucrose in the absence of primer, was measured after a 1-h preincubation with the respective serum. Under these conditions, *S. sobrinus* GTF$_{Ss}$ synthesized 1,976 ± 35 cpm of ^{14}C-total glucan in the absence of inhibitor, approximately three-fourths of which was associated with water-insoluble glucan. Percentage inhibition was expressed relative to the mean counts per minute incorporated into water-insoluble or water-soluble glucan synthesized by GTF after preincubation with eight sham-injected rat serum samples as follows: mean ± standard deviation of [1 − (mean replicate cpm of an individual rat anti-CAT or anti-GTF serum sample/mean cpm of the eight sham-injected rat serum samples)] × 100.
[c] Expression of significance is based on a comparison of counts per minute of GTF preincubated with immune sera with counts per minute of GTF preincubated with sham sera by using one-way analysis of variance and the Tukey-Kramer multiple comparisons test.

TABLE 4. Inhibition of *S. sobrinus* GTF-Sd-mediated formation of water-insoluble glucan (WIG) or water-soluble glucan (WSG) by mouse MAbs to CAT or GTF

MAb[a]	[^{14}C]glucan cpm (% inhibition)[b]	
	WIG	WSG
IgM MAb$_{CAT}$	1,342 ± 9 (35)	4,125 ± 25 (35)
IgM MAb TEPC 183	2,063 ± 3	6,450 ± 100
IgG MAb$_{GTF}$	426 ± 23 (44)	4,136 ± 10
IgG MAb MOPC 21	760 ± 15	4,102 ± 100
IgM MAb$_{GTF}$	1,670 ± 106 (19)	6,450 ± 50
IgM MAb TEPC 183	2,063 ± 3	6,211 ± 261

[a] MAbs were each tested at approximately 150 µg/ml (approximately 1.5 to 3.0 µg per assay).
[b] [^{14}C]glucan values are means ± standard deviations. Percentage inhibition of the formation of water-insoluble or water-soluble glucan was expressed as [1 − (mean cpm of the CAT or GTF MAb/mean cpm of the irrelevant MAb)] × 100. The indicated percentages of inhibition were significant at the P of <0.05 to <0.01 level.

GTF (Table 3). These rat sera gave relatively low but significant inhibition of both water-insoluble (17.0% ± 4.2% [mean ± standard error]) and water-soluble (10.0% ± 3.0%) glucan formation by GTF$_{Ss}$.

MAb reagents (IgM MAb$_{CAT}$, IgM MAb$_{GTF}$, and IgG MAb$_{GTF}$) were also tested for their ability to inhibit GTF activity. Representative experiments are shown in Tables 4 and 5. The anti-CAT IgM MAb significantly inhibited the formation of filter-retained glucan by both *S. sobrinus* GTF-I and GTF-Sd. GTF-I activity was inhibited by the anti-CAT IgM MAb in a dose-dependent manner similar to that of a rat antiserum to *S. sobrinus* GTF (Fig. 2A). MAb-based inhibition could be detected at concentrations of less than 50 ng per assay. Modest inhibition of water-soluble glucan synthesis by *S. sobrinus* GTF-Sd and *S. mutans* GTF was also observed (Tables 4 and 5).

The control (TEPC 183) and anti-CAT IgM MAbs were reduced, alkylated, and retested in the GTF-I inhibition assay at concentrations of 13 µg/ml. The anti-CAT IgM monomers, obtained after gel filtration on Superose 6, inhibited the formation of 16% of the filter-retained glucan activity by *S. sobrinus* GTF-I, compared with essentially no inhibition by the

TABLE 5. Inhibition of *S. sobrinus* GTF-I-mediated formation of water-insoluble glucan (WIG) or *S. mutans* GTF-mediated formation of water-soluble glucan (WSG) by mouse MAbs to CAT or GTF

MAb[a]	[^{14}C]glucan cpm (% inhibition)[b]	
	S. sobrinus GTF-I-mediated WIG	*S. mutans* GTF-mediated WSG
IgM MAb$_{CAT}$	4,050 ± 173 (49)	2,812 ± 74 (13)
IgM MAb TEPC 183	7,069 ± 240	3,213 ± 25
IgG MAb$_{GTF}$	2,970 ± 25 (18)	1,479 ± 10 (20)
IgG MAb MOPC 21	3,602 ± 20	1,825 ± 25
IgM MAb$_{GTF}$	3,459 ± 239 (51)	3,350 ± 50
IgM MAb TEPC 183	7,069 ± 240	3,213 ± 25

[a] MAbs were each tested at approximately 150 µg/ml (approximately 1.5 to 3.0 µg per assay).
[b] [^{14}C]glucan values are means ± standard deviations. Percentage inhibition of the formation of water-insoluble glucan or water-soluble glucan was expressed as [1 − (mean cpm of the CAT or GTF MAb/mean cpm of the irrelevant MAb)] × 100. The indicated percentages of inhibition were significant at the P of <0.05 to <0.01 level.
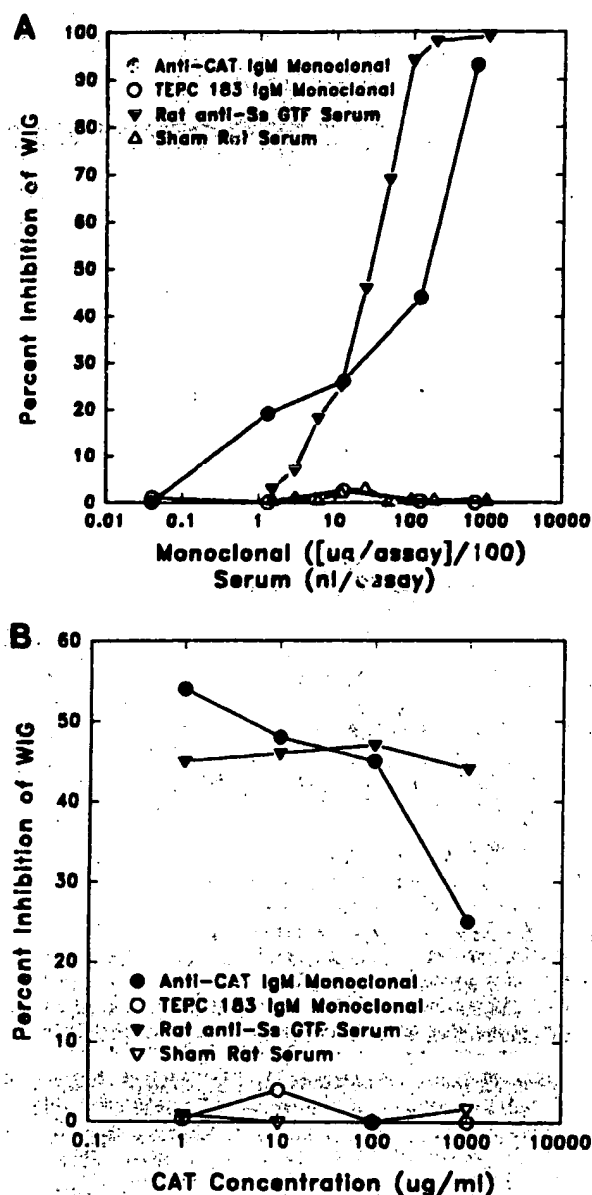
A

B

CAT Concentration (ug/ml)

FIG. 2. (A) Percentage inhibition of GTF-I-mediated water-insoluble glucan formation by anti-CAT mouse IgM MAb (closed circles) or serum from a rat injected four times with S. sobrinus GTF (closed triangles) as described in Materials and Methods. Various amounts (micrograms per assay per 100) of IgM MAb to CAT or control IgM MAb (TEPC 183) were assayed for the ability to inhibit [14C]glucose-labelled water-insoluble glucose formation from [14C-glucose]sucrose by S. sobrinus GTF-I. Various amounts (nanoliters of serum per assay) of sera from S. sobrinus GTF-immunized (closed triangles) or sham-immunized (open triangles) rats were also assayed for the ability to inhibit water-insoluble glucan formation. (B) Assay of the ability of the CAT peptide to competitively inhibit mouse anti-CAT IgM MAb reagent, TEPC 183 IgM MAb of irrelevant specificity, CAT polyclonal rat anti-GTF$_{Ss}$ serum, or sham-injected rat serum. Each of the above serum samples or MAb reagents were preincubated with the four indicated concentrations of the CAT construct prior to incubation with S. sobrinus GTF-I and then assayed for water-insoluble [14C]glucan formation. The mouse IgM MAb$_{CAT}$ and rat anti-GTF$_{Ss}$ serum inhibited 54 and 45% of the activity of S. sobrinus GTF-I, respectively, in the absence of CAT. No significant inhibition was observed with either the control mouse MAb (TEPC 183) or the rat polyclonal (sham

control IgM monomer. Although lower than the inhibition with a similar concentration of intact IgM MAb$_{CAT}$ (approximately 25%) (Fig. 2A), the presence of monomer-based inhibition indicated that the IgM-mediated effects were not simply a function of the pentameric configuration and size.

IgM MAb$_{GTF}$ and IgG MAb$_{GTF}$ reactive with epitopes on intact GTF also inhibited the formation of filter-retained glucan by both S. sobrinus GTF-I and GTF-Sd activity (Tables 4 and 5). However, these igG MAbs showed little or no inhibition of water-soluble glucan formation by S. sobrinus GTF-Sd or S. mutans GTF.

The specificity of the reaction of the immune sera and MAbs was further tested by preincubating the rat antiserum to GTF$_{Ss}$ and the IgM and IgG MAb reagents with increasing concentrations of the CAT construct (Fig. 2B). This pretreatment removed detectable GTF-inhibitory activity from the IgM anti-CAT MAb. No significant reduction in S. sobrinus GTF-I inhibition was observed by pretreatment of a rat antiserum to GTF$_{Ss}$ with the CAT construct at concentrations as high as 1 mg/ml.

DISCUSSION

Molecular targets for potential immunological interference with GTF activity should be conformationally accessible to permit binding to antibody. The sequence DANFDSIRV-DAVDNVDADLLQ, selected for construct preparation and for immunization in this study, apparently shares an accessible epitope(s) with native GTF since antisera from rats injected with CAT, and MAb derived from similarly immunized mice, showed a substantial reaction with S. sobrinus GTF (Fig. 1; Table 2).

Another consideration in the selection of sequences for a potential subunit vaccine to achieve the broadest protective effect would be that target epitopes are shared on GTFs from all mutans streptococcal species that infect humans. Much of the N-terminal sequence of mutans streptococcal GTF is highly conserved (1, 6, 7, 9, 11, 25). In fact, the sequence reported in Table 1 differs by no more than two residues from the sequences of GTF-Is from mutans streptococci or Streptococcus salivarius. Thus, it could be expected that if the CAT peptide sequence includes an epitope(s) that is also sequentially defined on GTF, then a similar epitope(s) could be expected to be found on the GTF-I sequence of several species. Support for this hypothesis is seen in the reactions of both the rat polyclonal antibodies and mouse MAbs to CAT with S. sobrinus and S. mutans GTF (Fig. 1; Table 2).

Although antibody binding to functionally important domains may not be necessary theoretically to achieve immunological protection, binding to catalytic domains should result in a loss of enzyme activity The sequence DANFDSIRVDAVD NVDADLLQ contains an aspartic acid that has been implicated in an early catalytic step of glucan synthesis (19, 20). Rat serum antibody (Table 3) and mouse IgM MAb (Tables 4 and 5) to the CAT construct both significantly inhibited the ability of S. sobrinus GTF to synthesize glucan. The mouse IgM anti-CAT MAb (IgM MAb$_{CAT}$) was shown to inhibit GTF-I activity at concentrations as low as 50 ng per assay (Fig. 2). The pentameric structure was not required for inhibition by the

rat serum) reagent at any CAT concentration. Each datum point in the figure represents the mean of at least one assay performed in duplicate. Individual serum replicates varied by no more than 4.7% from the mean (mean variation, 1.9%).

IgM MAb. The water-insoluble glucan product of *S. sobrinus* GTF-Sd, whose activity is also dependent n the presence of dextran primer, as also inhibited by the MAb. The multiple specificities in polyclonal rat antibody to *S. sobrinus* GTF$_{Ss}$ gave the broadest range of inhibition, although the synthesis of water-soluble glucan by GTF-Sd was least affected (data not shown).

We have recently shown that antibody to a repeated sequence in the C-terminal third of GTF (GLU) could also inhibit GTF activity (24). This sequence has been associated with glucan-binding properties of GTF (21, 22). The pattern of GTF inhibition by antibody to GLU differed from that by antibody to CAT. Only the formation of water-insoluble glucan by *S. sobrinus* GTF-Sd was inhibited by rat anti-GLU antibody under the conditions of the assay. In contrast, the formation of water-insoluble glucan by both *S. sobrinus* GTF-I and GTF-Sd were the principal activities inhibited by the mouse IgM MAb to CAT. Rat antisera to CAT modestly inhibited the formation of water-soluble and water-insoluble glucan from *S. sobrinus* GTF mixtures. The mouse IgM MAb$_{CAT}$ also inhibited *S. mutans* GTF, although the level of inhibition was low. This interspecies inhibition pattern by the anti-CAT antibody may be due to the fact that the sequences corresponding to the CAT peptide are more highly conserved than those sequences corresponding to the GLU peptide. However, Chia et al. (2) found that MAb directed to a 19-mer sequence in the N-terminal third of *S. mutans* GTF inhibited the GTF-C but not the GTF-D isozyme, despite sequence identity in the respective regions of these two enzymes. These observations suggest that sequence homology is only one of several structural attributes that dictate epitopic structure among mutans streptococcal GTFs.

Cope and Mooser (4) have reported that rabbit IgG antibody to two 22-mer peptides reacted only weakly with native GTF and did not inhibit *S. sobrinus* GTF-I or GTF-S, although their primary sequences were nearly identical to that of CAT. They suggested that these 22-mer peptides may not lie on the surface of GTF, thus limiting access of potentially inhibitory antibody to the eliciting peptide. Although this may be the case, these authors (4) used a monovalent peptide, unbound to carrier, for immunization, which may have limited the immunogenicity of the peptide. In the present study, the CAT peptide was synthesized as a quatravalent construct on a lysine backbone. The construct used in our studies resulted in the formation of rat antibody that reacted with native *S. sobrinus* and *S. mutans* GTF in an ELISA, and some of the more-high-titered sera significantly inhibited *S. sobrinus* GTF activity. The peptide configurations adopted by the monovalent (4) and quatravalent multiple antigenic peptide constructs, and the epitopes created by these configurations, are likely to differ in solution and give rise to antibody that differs in amount, specificity, and/or affinity. The genetic backgrounds of rats, mice, and rabbits may also contribute to variations in immune response parameters that lead to the formation of antibody.

Thus, antibody to epitopes present in the N-terminal (CAT) and C-terminal (GLU) regions of GTF apparently can interfere with enzyme function. In addition, Dertzbaugh and Macrina (5) have demonstrated inhibition of GTF activity with antibody to a chimeric peptide that included a sequence closer to the N terminus (residues 342 to 356) than the CAT sequence (residues 487 t 507) in *S. mutans* GTF-C (12, 29). Also, Chia and coworkers (3) have synthesized a 19-mer peptide, hom logous to a sequence nearly adjacent t the CAT region, that contained an epitope abl to remove some of the GTF-inhibit ry activity f polyclonal antibodies directed t *S. mutans* GTF-B and GTF-C. These observati ns suggest that

inhibition of GTF activity can be achieved by the binding of antibody to sequences that may n t necessarily be associated with functi n. Although the anti-peptide antibodies are intended t be directed to sequential epitopes, antibody binding potentially could modify enzyme function, not only by directly blocking access of substrate to functional domains, but also through conformational changes that affect catalytic activity.

These studies suggest that subunit vaccines that are based on GTF sequences have the potential to modify dental caries caused by mutans streptococci. Antibody-mediated inhibition of the caries process could be achieved by interference with GTF catalytic activity, by modification of the nature of the glucan product, or by agglutination of microorganisms through bacterial cell surface GTF epitopes. The success of such a vaccine strategy is predicated on the incorporation of an appropriate peptide(s) into a carrier construct that results in an effective immune response.

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AB   Competitive time-resolved fluoroimmunoassays (FIAs) were developed for
      measuring 1,3,5(10)-estratrien-3-ol-17-one glucosiduronate (estrone
      3-glucuronide, E-13G) and 5-beta-Pregnane-3-alpha,20-alpha-diol
      3-glucosiduronate (pregnanediol 3-glucuronide, Pd3G) in unextracted
urine.
      The assays are specific, detect 0.98 ng E-13G/mL and 0.035 mu-g Pd3G/mL,
      measure 102.8 +- 2.0% of E-13G and 93.6 +- 2.9% of Pd3G added, and
exhibit
      between and within **assay** coefficients of variation,
      respectively, of 5.3% and 7.1% for E-13G and 6.8% and 7.8% for Pd3G. The
      urine matrix does not interfere with the **assay**. Urinary steroid
      glucuronide profiles measured by these FIAs conform to those of urinary
      steroid glucuronides and serum estradiol and progesterone measured by
      other established immunoassays. These FIAs afford the advantages of
      non-radioisotopic procedures and urine sample collection (convenience,
      non-invasiveness, integration of pulsatile secretion) to evaluate
      menstrual function in epidemiological, medical, and athletic populations.

L7   ANSWER 3 OF 9 CAPLUS COPYRIGHT 2003 ACS
AN   2003:242606 CAPLUS
DN   138:232191
TI   Qualitative evaluation of ovarian cycle in relation to impaired human
      fertility
IN   **Coley, John**; Miro, Fernado; Aspinall, Laurence John; Murray,
      Peter George; Dadd, Anthony William; Zhang, Zhi Gang; Mundill, Paul
PA   Inverness Medical Switzerland G.m.b.H., Switz.
SO   PCT Int. Appl., 54 pp.
      CODEN: PIXXD2
DT   Patent
LA   English
FAN.CNT 1
      PATENT NO.   KIND DATE   APPLICATION NO. DATE
      --------------- ---- -------   --------------- -------
PI   WO 2003025582   A1   20030327   WO 2002-GB4235   20020918
         W: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN,
             CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, ES, FI, GB, GD, GE, GH,
             GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR,
             LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, OM, PH,
             PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TN, TR, TT, TZ,
             UA, UG, US, UZ, VN, YU, ZA, ZM, ZW, AM, AZ, BY, KG, KZ, MD, RU,
             TJ, TM
         RW: GH, GM, KE, LS, MW, MZ, SD, SL, SZ, TZ, UG, ZM, ZW, AT, BE, BG,
             CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL,
             PT, SE, SK, TR, BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR,
             NE, SN, TD, TG
PRAI EP 2001-307912   A   20010918
AB   Claimed is a method of detecting a retarded cycle in a human female
      subject experiencing ovarian cycle disorders. The method comprises the
      steps of: obtaining a sample of body fluid from the subject on each of a
      plurality of, but not less than three, days; testing each of the
plurality
      of samples to det. the concn. therein of at least one analyte of
      significance in the ovulatory cycle; comparing a result detd. from said

testing with a predetd. threshold value; and, if said detd. result is different from the threshold value; declaring the cycle, during which the samples were taken, to be a retarded cycle. Also claimed is the use of the method to detect the menopause transition as well as to identify impaired ovarian function in relation to fertility disorders.

RE.CNT 6   THERE ARE 6 CITED REFERENCES AVAILABLE FOR THIS RECORD
            ALL CITATIONS AVAILABLE IN THE RE FORMAT

L7   ANSWER 4 OF 9 CAPLUS COPYRIGHT 2003 ACS
AN   2001:747245 CAPLUS
DN   135:252338
TI   Test methods and devices for analyte isoforms
IN   **Butlin, Lorraine Diane; Coley, John; Eida,
     Stephen James; Gani, Mohamed Mutwahar**
PA   Unilever PLC, UK; Unilever N.V.
SO   Eur. Pat. Appl., 8 pp.
     CODEN: EPXXDW
DT   Patent
LA   English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI EP 1143250	A2	20011010	EP 2001-303132	20010402
R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT, IE, SI, LT, LV, FI, RO				
JP 2001349892	A2	20011221	JP 2001-103658	20010402
CA 2343488	AA	20011003	CA 2001-2343488	20010403
US 2002042149	A1	20020411	US 2001-828624	20010403
PRAI EP 2000-302810	A	20000403		

AB   A method and test device for differentiating between states of an analyte that can exist in different forms, such as FSH (FSH). The method or test device uses two contemporaneous assays, the first of which does not differentiate between the two analyte states and the second of which does,
     and the **assay** results are compared. A novel pair of anti-FSH monoclonal antibodies that can be used together in a sandwich-format **assay** to differentiate premenopausal and postmenopausal FSH samples is disclosed.

L7   ANSWER 5 OF 9 CAPLUS COPYRIGHT 2003 ACS
AN   2001:747244 CAPLUS
DN   135:252337
TI   Test methods and devices for analyte isoforms
IN   **Butlin, Lorraine Diane; Coley, John; Eida,
     Stephen J.; Gani, Mohamed M.**
PA   Unilever PLC, UK; Unilever N.V.
SO   Eur. Pat. Appl., 9 pp.
     CODEN: EPXXDW
DT   Patent
LA   English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE

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PI     EP 1143249        A2    20011010      EP 2001-303130   20010402
            R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LI, LU, NL, SE, MC, PT,
                IE, SI, LT, LV, FI, RO
       CA 2342683         AA    20011003      CA 2001-2342683  20010402
       JP 2001349891      A2    20011221      JP 2001-103646   20010402
       US 2002045273      A1    20020418      US 2001-824587   20010403
PRAI   EP 2000-302811     A     20000403
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AB   A method and test device for differentiating between states of an analyte
     that can exist in different forms, such as FSH. The method or test
device
     uses a pair of specific binding agents, esp. monoclonal antibodies, in
two
     assays for the same analyte. The assays, applied to contemporaneous
     samples, differ from one another in format, one being a two step
     assay and the other being one step. A novel pair of anti-FSH
     monoclonal antibodies that can be used together in two such assays to
     differentiate pre-menopausal and post-menopausal FSH samples is
disclosed.

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L7     ANSWER 6 OF 9   CAPLUS   COPYRIGHT 2003 ACS
AN     1994:262308   CAPLUS
DN     120:262308
TI     Contraceptive method and test kit for urinary LH assay
IN     Coley, John; Davis, Paul James; Senior, Stephanie Jane
PA     Unipath Ltd., UK; Unilever PLC; Unilever N. V.
SO     PCT Int. Appl., 28 pp.
       CODEN: PIXXD2
DT     Patent
LA     English
FAN.CNT 1
       PATENT NO.          KIND  DATE          APPLICATION NO.   DATE
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PI     WO 9404928          A1    19940303      WO 1993-EP2144    19930810
            W:  AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP,
                KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD,
                SE, SK, UA, US, VN
            RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE,
                BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG
       AU 9349473          A1    19940315      AU 1993-49473     19930810
       EP 656122           A1    19950607      EP 1994-908163    19930810
       EP 656122           B1    19970212
            R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, NL, PT, SE
       EP 745854           A1    19961204      EP 1996-112236    19930810
       EP 745854           B1    20011205
            R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, NL, PT, SE
       AT 148947           E     19970215      AT 1994-908163    19930810
       ES 2098925          T3    19970501      ES 1994-908163    19930810
       BR 9306938          A     19990112      BR 1993-6938      19930810
       AT 210299           E     20011215      AT 1996-112236    19930810
       ES 2169179          T3    20020701      ES 1996-112236    19930810
       AU 9852066          A1    19980319      AU 1998-52066     19980114
PRAI   GB 1992-17808       A     19920821
       EP 1994-908163      A3    19930810
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WO 1993-EP2144   W   19930810
AB   The likelihood of conception is reduced in humans by measuring the urinary
      LH concn. frequently (preferably daily) beginning on a predetd. day
      (between days 5 and 15 after the onset of menses) until the LH surge
      assocd. with ovulation is detected, and avoiding unprotected intercourse
      during the period of LH testing and until .gtoreq.2 days after LH surge
      detection. A test kit contains reagent-impregnated immunochromatog.
      assay strips incorporating a labeled anti-LH antibody, a measuring
      device that signals the user when the urinary LH concn. is .gtoreq.20-30
      mIU/mL, and a chart or other recording means for entering daily LH
levels.
      The chart can be used to predet. an optimum test starting day for the
      subsequent cycle.

L7   ANSWER 7 OF 9 CAPLUS COPYRIGHT 2003 ACS
AN   1994:262307 CAPLUS
DN   120:262307
TI   Ovulation monitoring method and kit
IN   Catt, Michael; **Coley, John**; Davis, Paul James
PA   Unipath Ltd., UK; Unilever PLC; Unilever N. V.
SO   PCT Int. Appl., 38 pp.
      CODEN: PIXXD2
DT   Patent
LA   English
FAN.CNT 1

PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI WO 9404924	A1	19940303	WO 1993-EP2146	19930810

      W: AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP,
          KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD,
          SE, SK, UA, US, VN
      RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE,
          BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG

AU 9347092	A1	19940315	AU 1993-47092	19930810
EP 656118	A1	19950607	EP 1993-917783	19930810
EP 656118	B1	19970212		

      R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, NL, PT, SE

JP 08500669	T2	19960123	JP 1993-505868	19930810
EP 745853	A1	19961204	EP 1996-112234	19930810
EP 745853	B1	20011107		

      R: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, NL, PT, SE

AT 148945	E	19970215	AT 1993-917783	19930810
ES 2100555	T3	19970616	ES 1993-917783	19930810
JP 10185914	A2	19980714	JP 1998-1883	19930810
BR 9306939	A	19990112	BR 1993-6939	19930810
RU 2146059	C1	20000227	RU 1995-106780	19930810
JP 3149111	B2	20010326	JP 1994-505868	19930810
JP 2001178729	A2	20010703	JP 2000-349877	19930810
AT 208503	E	20011115	AT 1996-112234	19930810
ES 2167492	T3	20020516	ES 1996-112234	19930810
FI 9500717	A	19950217	FI 1995-717	19950217
NO 9500635	A	19950420	NO 1995-635	19950220

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        AU 9856318        A1    19980521      AU 1998-56318      19980219
PRAI  GB 1992-17866     A     19920821
      EP 1993-917783    A3    19930810
      JP 1994-505868    A3    19930810
      WO 1993-EP2146    W     19930810
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AB   A method of monitoring the status of a current ovulation cycle of a human
      female involves repeated testing of the body fluid concn. of .gtoreq.1
      analyte of significance in relation to the status of the ovulation cycle,
      e.g. urinary estrone 3-glucuronide or estradiol, during at least the
      preovulation phase of the current ovulation cycle. Testing, e.g. by EIA,
      is preferably commenced .gtoreq.5 days following the onset of menses but
      .gtoreq.2 days in advance of the earliest day on which ovulation occurred
      in .gtoreq.1 previous ovulation cycle in the same individual. A kit for
      the **assay** includes an electronic device which measures and
      stores optical signal data from the **assay** and compares them with
      data from previous cycles.

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L7    ANSWER 8 OF 9   CAPLUS   COPYRIGHT 2003 ACS
AN    1994:262305   CAPLUS
DN    120:262305
TI    Ovulation monitoring method, device, and kit
IN    Catt, Michael; Coley, John; Davis, Paul James
PA    Unilever PLC, UK; Unilever N. V.; Unipath Ltd.
SO    PCT Int. Appl., 58 pp.
      CODEN: PIXXD2
DT    Patent
LA    English
FAN.CNT 1
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	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
PI	WO 9404926	A1	19940303	WO 1993-EP2148	19930810

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      W:  AT, AU, BB, BG, BR, BY, CA, CH, CZ, DE, DK, ES, FI, GB, HU, JP,
          KP, KR, KZ, LK, LU, MG, MN, MW, NL, NO, NZ, PL, PT, RO, RU, SD,
          SE, SK, UA, US, VN
      RW: AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE,
          BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG
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	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
	AU 9347094	A1	19940315	AU 1993-47094	19930810
	EP 656120	A1	19950607	EP 1993-917785	19930810
	EP 656120	B1	19970212		

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      R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, NL, PT, SE
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	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
	JP 08500671	T2	19960123	JP 1993-505870	19930810
	JP 2755823	B2	19980525		
	EP 754949	A1	19970122	EP 1996-112233	19930810

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      R:  AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LI, NL, PT, SE
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	PATENT NO.	KIND	DATE	APPLICATION NO.	DATE
	AT 148946	E	19970215	AT 1993-917785	19930810
	ES 2100556	T3	19970616	ES 1993-917785	19930810
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AB   A simple and convenient device is described for carrying out chem. or
      clin. testing using specific binding assays at the patient's bedside or
      home which comprises a test component having a sensitized solid surface
      carrying an immobilized component of a specific binding pair relevant to
      the **assay,** an accessory removable component carrying an
      accessory solid surface, a container for sample liqs., and a handle. The
      sample contacts the sensitized surface and the accessory surface acts to
      retain the liq. in contact even after removal of the device from further
      contact with the source. After removal of the accessory component, the
      sensitized surface is left exposed and accessible to further liq.
      treatments (e.g., washings and/or reagents).

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7/25/03

(54) **MATERIALS AND METHODS FOR DETECTION AND QUANTITATION OF AN ANALYTE**

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(57) **ABSTRACT**

The subject invention pertains to methods and materials for accurately assessing the presence or absence of analytes of interest in samples, particularly in physiological samples. The subject invention involves utilizing a ligand binding domain (LBD) of a receptor to selectively capture the analyte target specific for that LBD. In one embodiment, the receptor is a protein or polypeptide. The ligand binding domain is allowed to react with a sample and the presence or amount of ligand (i.e., target analyte) bound by the LBD is determined. Suitable analytes include soluble analytes such as hormones, enzymes, lipoproteins, bacterial or viral antigens, immunoglobulines, lymphokines, cytokines, drugs, soluble cancer antigens, and the like. The methods of the present invention can be performed in both liquid-phase and solid-phase.

FIG. 1

FIG. 2

FIG. 3A

LEGEND

Serum constituents

Solid support, e.g. plastic, nitrocellulose, DEAE

eER-LBD

17ß-estradiol

Sepharose

Colorimetric enzyme, e.g. HP

Enzyme substrate

Precipitable enzyme product

FIG. 3B   Add serum to eER-LBD-enzyme conjugate

FIG. 3C   Incubate to allow ligand binding

FIG. 3D   Apply to estrogen affinity matrix

FIG. 3E   Wash matrix

FIG. 3F   Add substrate and allow color development

"flow through"

FIG. 4A

LEGEND

 Serum constituents

_____ Solid support, e.g. plastic, nitrocellulose, DEAE

 eER-LBD

 17ß-estradiol

● Sepharose

ʊ Colorimetric enzyme, e.g. HP

● Enzyme substrate

✳ Precipitable enzyme product

FIG. 4D Add estrogen-enzyme conjugate

FIG. 4E Add substrate and allow color development

FIG. 4B Incubate serum with solid-phase eER-LBD

Coated vessel
Dipstick
Filtration Chamber
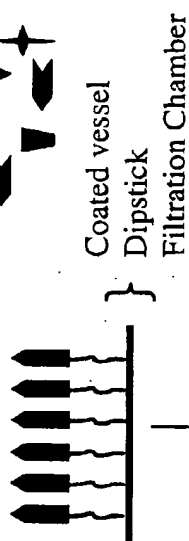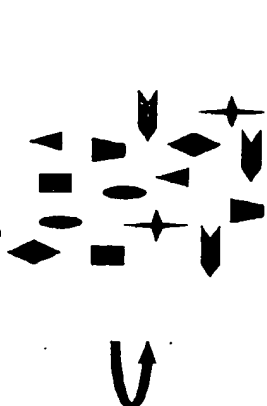
FIG. 4C Evacuate and wash solid-phase eER-LBD

Wash or filtrate

Estrogen (target analyte)

Other constituents in biological fluid

Estrogen receptor ligand binding domain

Colored latex beads

Estrogen affinity adsorbent, i.e. estradiol-17ß covalently linked to a solid phase support such as nitrocellulose

Solid-phase capture agent, e.g. streptavidin, anti-estrogen receptor antibodies, anti-BSA antibodies linked to a solid phase support

FIG. 5A

1. Sample Addition

2. Conjugate release

3. eER-LBD interaction
with estrogen

Adsorption Zone

Conjugate Zone
(Latex-eER-LBD)

4. Adsorption of latex beads
with unoccupied eER-LBD

Capture Zone

5. Capture of latex beads
with occupied eER-LBD

FIG. 5B

Sample Application Well

Zone 1. Conjugate Resorption

Zone 2. Adsorption

Zone 3. Analyte Capture

FIG. 5C

MATERIALS AND METHODS FOR DETECTION AND QUANTITATION OF AN ANALYTE

CROSS-REFERENCE TO A RELATED APPLICATIONS

[0001] This application is a continuation of U.S. application Ser. No. 09/660,979, filed Sep. 13, 2000, now U.S. Pat. No. 6,500,629, which claims the benefit of U.S. Provisional Application No. 60/153,627, filed Sep. 13, 1999.

BACKGROUND OF THE INVENTION

[0002] There are a number of assay systems available for detection and quantitation of analytes, particularly analytes of biological interest. Current assay systems include enzyme immunoassay (EIA), radioimmunoassay (RIA), and enzyme linked immunosorbent assay (ELISA). Among the analytes frequently assayed with such systems are: 1) hormones, such as human chorionic gonadotropin (hCG), frequently assayed in urine as a marker of human pregnancy; 2) antigens, particularly antigens specific to bacterial, viral, and protozoan pathogens, such as Streptococcus and hepatitis virus; 3) antibodies, particularly antibodies induced as a result of infection with pathogens, such as antibody to the bacterium *Heliobacter pylori* and to human immunodeficiency virus (HIV); 4) enzymes, such as aspartite aminotransferase, lactate dehydrogenase, alkaline phosphatase, and glutamate dehydrogenase, frequently assayed as indicators of physiological function and tissue damage; 5) other proteins, such as hemoglobin, frequently assayed in determinations of fecal occult blood, an early indicator of gastrointestinal disorders such as colon cancer; 6) drugs, both therapeutic drugs, such as antibiotics, tranquilizers and anticonvulsants, and illegal drugs of abuse, such as cocaine, heroine, and marijuana; and 7) environmental pollutants such as pesticides and aromatic hydrocarbons and vitamins.

[0003] Such systems are frequently used by physicians and medical technicians for rapid in-office diagnosis and therapeutic monitoring of a variety of conditions and disorders. They are also increasingly used by patients themselves for at-home or on-site monitoring of such conditions and disorders.

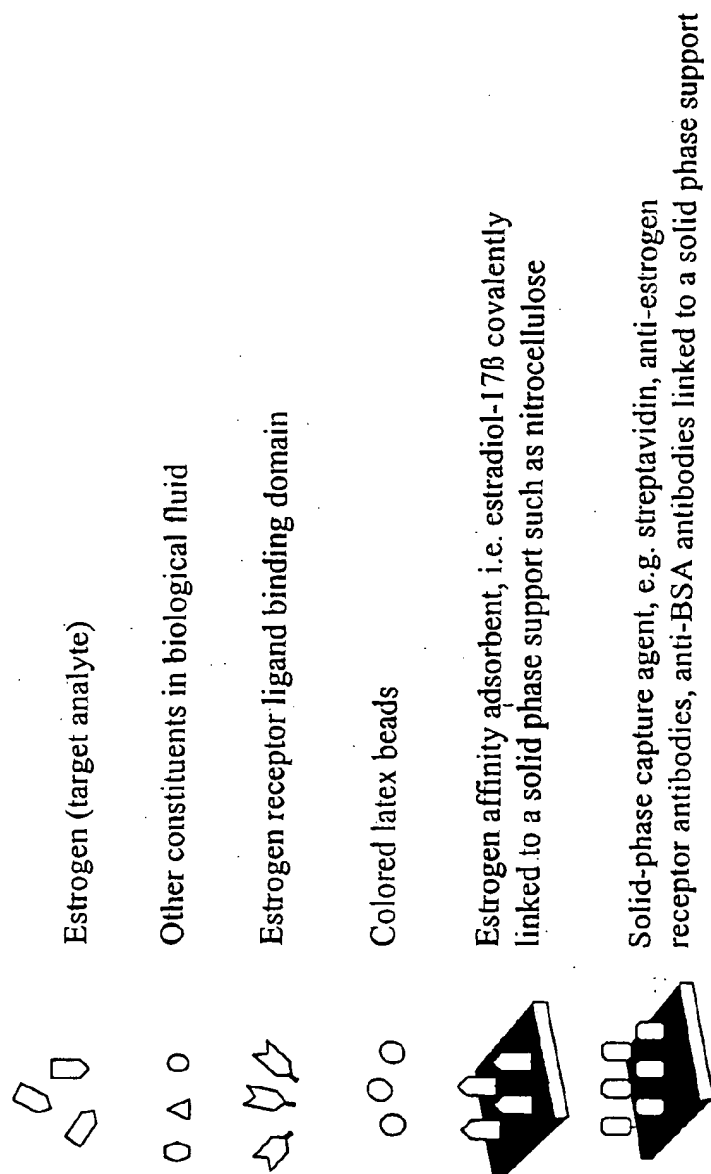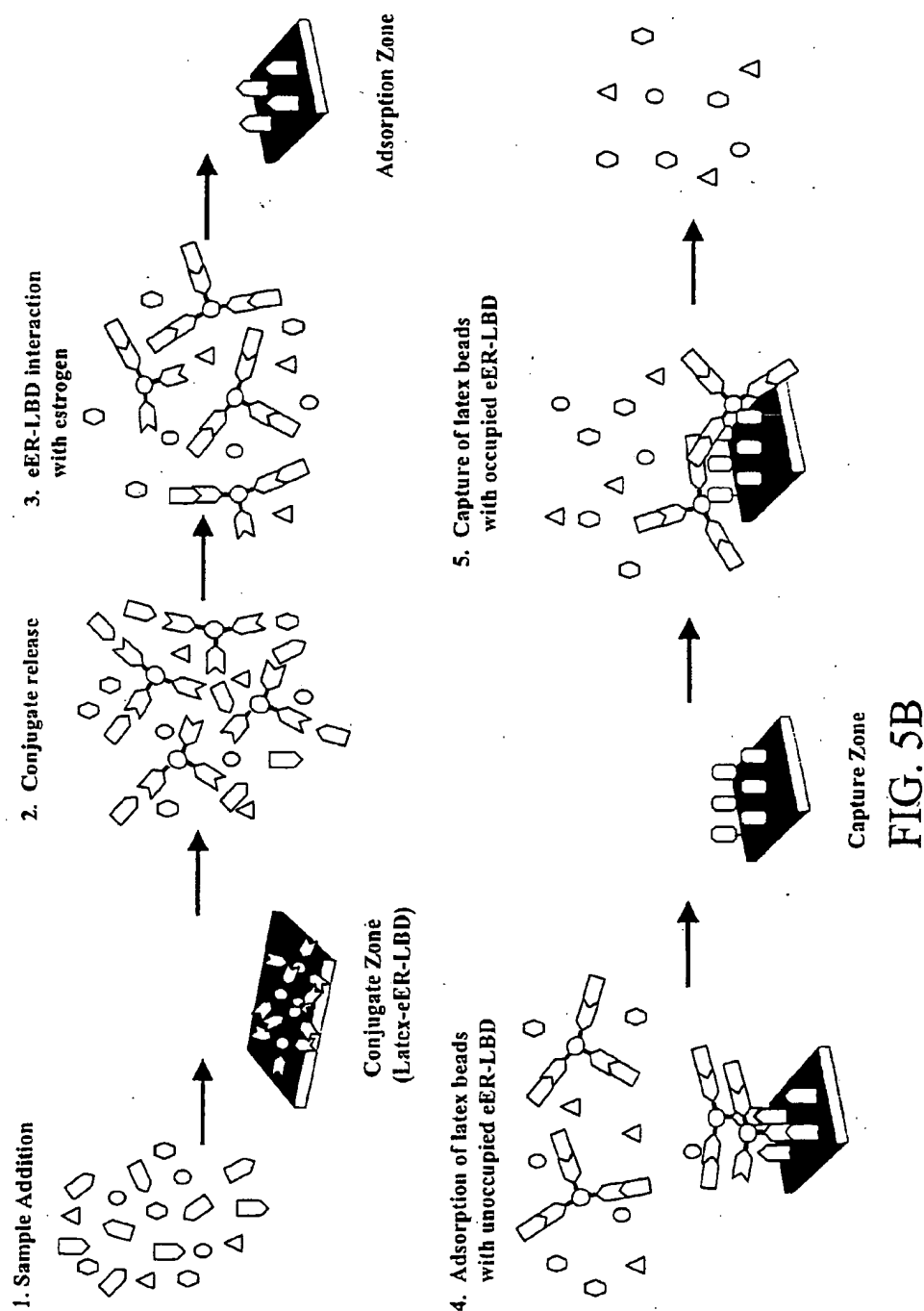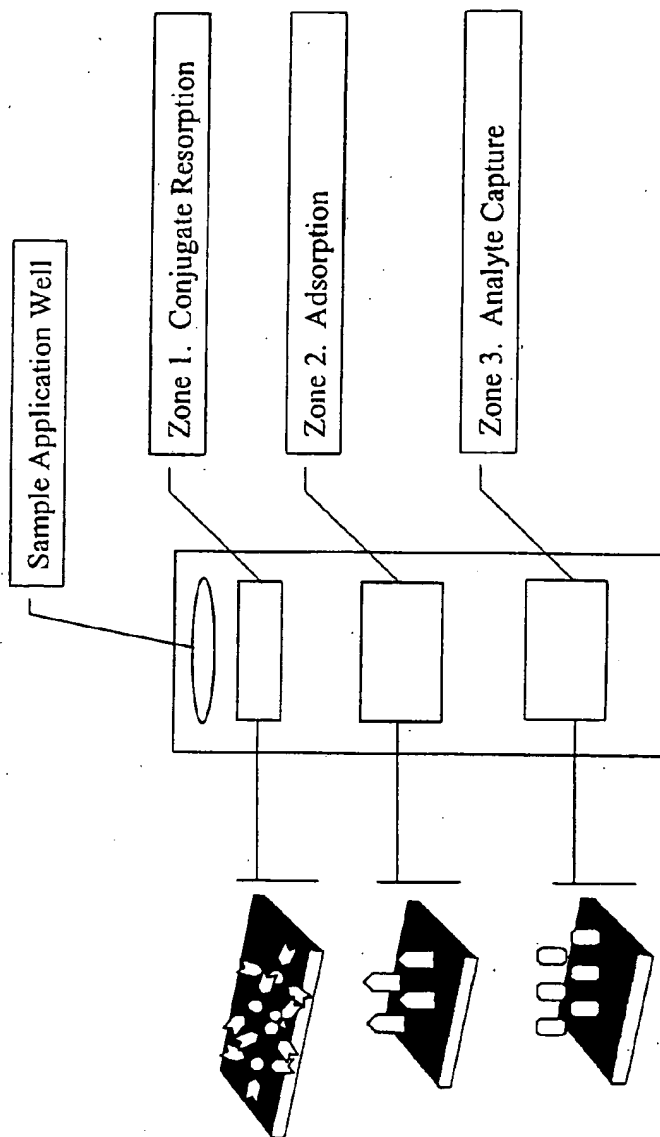[0004] Among the most popular of such assay systems are immunoassays, which depend on the specific interaction between an antigen or hapten and a corresponding antibody. The use of immunoassays as a means of testing for the presence and/or amount of clinically important molecules has been known for some time. As early as 1956, J. M. Singer reported the use of an immune-based latex agglutination test for detecting a factor associated with rheumatoid arthritis (Singer et al., 1956).

[0005] Development of the first radioimmunoassay by Rosalyn Yalow and Sol Berson (1959) set the stage for measurement of a wide variety of hormones in biological fluids by binding the hormone specifically and with high affinity to antibodies developed in animals against the hormone in question. The assay developed by Drs. Yalow and Berson employed antibodies formed against the protein hormone, insulin, and utilized a radiolabeled form of insulin as the marker, or "reporter" hormone. Antibodies became a useful way to "capture" a specific hormone from biological fluids and under conditions of constant antibody concentration and with some easily detected source of labeled hor-

mone (usually radioactively labeled; hence the name "radioimmunoassay") the amount of hormone "captured" from the biological fluid could be quantified by comparison to known concentrations of the hormone in similar conditions. In practice of the art, known amounts of (unlabeled) hormone, (insulin in the example) were allowed to compete for binding to the antibody with a known and fixed amount of I^{131} labeled insulin. The radiolabeled form of hormone, and the amount of antibody were held constant while the amount of unlabeled hormone was varied. This was the basis of a "standard curve" from which the amount of radioactive label that bound to the antibody varied inversely with the amount of unlabeled hormone. Comparison of the mass of unlabeled hormone required to displace a given amount of labeled hormone could then be used to estimate mass of an sample hormone. Separation of the fractions which were unreacted with the antibody (unbound) was carried out by a variety of chemical separation methods. In the original teaching of Yalow and Berson (1959), separation of the antibody-bound fractions of insulin from the unbound (free) fractions of insulin was carried by electrophoresis. Subsequent to their report, many means of separating bound from free fractions have been utilized, including column chromatography, salt or organic solvent precipitation of the protein (antibody), double antibody (in which the gamma globulin fraction of the species immunized against the hormone is then introduced to a different species to create an anti-antibody, or second antibody, and solid phase, in which the antibody is held by electrostatic forces to a solid interphase such as the inner wall of a test tube, flat disc, or elongate stick (dipstick) and separation of bound from free requires simple physical separation of the solid phase from the liquid phase containing the free fractions. A variant of the technique of radioimmunoassay involved coupling small, non-immunogenic molecules to larger, highly immunogenic molecules, such as bovine serum albumen (BSA), thyroglobulin (TG), or keyhole limpet hemocyanin (KLH) and stimulation of antibodies that recognized the smaller, non-immunogenic, portion of the hapten molecule. This modification of the technique permitted quantification of small hormones, such as steroids and prostaglandins.

[0006] While radioimmunoassay is a very useful tool for conducting research and for certain clinical applications, it has several drawbacks as far as practical management of endocrine or other hormonal states. A major drawback is the use of an antibody as a "capture protein." Development of polyclonal antibodies is accomplished by administering the hormone to an animal that regards it as foreign and develops antibodies against it. The process is very much trial and error and involves the use of a number of animals and screening of the antibody before determination of its usefulness. Once an ideal polyclonal antibody preparation has been obtained, the animal's plasma must be harvested and husbanded carefully, for once the animal dies, the supply of that particular antibody is lost forever.

[0007] The act of mounting an immune attack against a foreign protein and producing antibodies is actually a mixture, or collection of antibodies (hence the term "polyclonal antibody"), each of which is directed against a particular amino acid sequence called an epitope. A refinement of this process involves the production of monoclonal antibodies. Monoclonal antibodies are derived by collecting individual spleen cells from animals immunized by administration of a foreign protein and culturing the lymphocytes in vitro. The

cells are then screened to determine their binding characteristics, and those cells that possess appropriate binding are then cloned and maintained as an antibody-specific, continuous cell line. Thus, once appropriate cell cultures are obtained, they may be kept essentially indefinitely, thereby obviating one of the negative aspects of polyclonal antibodies.

[0008] However, monoclonal antibodies also have some drawbacks. For one thing, they are so specific as to be a detriment in some cases. Monoclonal antibodies are directed against amino acid sequences (epitopes) that are often common features of the tertiary structure of proteins. In this case the monoclonal antibodies are really not specific as one might believe at first. This drawback can be overcome by very stringent screening and validation of the assays utilizing monoclonal antibodies, but greater effort is often required. Additionally, monoclonal antibodies tend to be monovalent, which may restrict hierarchical or sandwich type coupling to other molecules for the purpose of separation or of amplification of the reporter signal.

[0009] Immunoassays fall into two principal categories: "sandwich" and "competitive," according to the nature of the antigen-antibody complex to be detected and the sequence of reactions required to produce that complex. Generally, the sandwich immunoassay calls for mixing the sample that may contain the analyte to be assayed with antibodies to the analyte. These antibodies are mobile and typically are linked to a detectable label or a disclosing reagent, such as dyed latex or a radioisotope. This mixture is then applied to a chromatographic medium containing a band or zone of immobilized antibodies to the analyte of interest. When the complex of the molecule to be assayed and the labeled antibody reaches the zone of the immobilized antibodies on the chromatographic medium, binding occurs and the bound labeled antibodies are localized at the zone. This indicates the presence of the molecule to be assayed. This technique can be used to obtain quantitative or semi-quantitative results. Examples of sandwich immunoassays performed on test strips are described by U.S. Pat. No. 4,168,146 to Grubb et al. and U.S. Pat. No. 4,366,241 to Tom et al., both of which are incorporated herein by reference.

[0010] In competitive immunoassays, the label is typically a labeled analyte or analyte analogue which competes for binding of an antibody with any unlabeled analyte present in the sample. Competitive immunoassays are typically used for detection of analytes such as haptens, each hapten being monovalent and capable of binding only one antibody molecule. Examples of competitive immunoassay devices are those disclosed by U.S. Pat. No. 4,235,601 to Deutsch et al., U.S. Pat. No. 4,442,204 to Liotta, and U.S. Pat. No. 5,208,535 to Buechler et al., all of which are incorporated herein by reference.

[0011] Although useful, currently available immunoassay techniques have a number of disadvantages. For example, because antibodies and other immunoassay reagents are susceptible to environmental conditions, at-home or on-site methods are problematic. Further, antibodies continue to be expensive to produce. Accordingly, it would be advantageous to employ an analyte assay system with all of the advantages of an immunoassay, but which is free of the inherent disadvantages traditionally associated with such immunologically based systems.

[0012] In addition to immunoassays, protein-binding assays have been utilized to quantitatively detect various analytes within a sample. These assay systems utilize a protein, such as a protein receptor, which is specific for a particular analyte target. Unfortunately, because these systems utilize the entire protein and the protein may have binding sites for more than one target analyte, there can be problems with cross-reactivity and assay accuracy. For example, in angiotensin II SPA receptor binding assays, the whole angiotensin membrane protein receptor is immobilized on a bead. This bead-receptor complex is then contacted with a sample, binding any angiotensin present in the sample.

[0013] Determination of optimum breeding time is important to the success of breeding domestic species. For example, the ability to rapidly and accurately measure equine ovarian steroid hormone estradiol (E2), in the field, would greatly benefit veterinarians in assisting managers of equine breeding farms in making breeding management decisions. The literature is replete with reference to early pregnancy failure in a variety of domestic species, e.g. sheep, 20 to 30% (Edey, 1969; Hanley, 1961; Nancarrow, 1994), goats, 6 to 42% (Kidder et al., 1954; Diskin et al., 1980), pigs, 20 to 30% (Bolet, 1986), cattle, 8 to 42% (Pope et al., 1985) and mares, 15 to 25% (Ginther, 1986; Villahoz et al., 1985; Ball et al., 1986; Ball et al., 1987). A major component of these losses represents errors of fertilization and/or exchange of genetic information (Hunter, 1994; King, 1990) or errors in the interaction between the maternal uterine unit and the developing conceptus (Bazer et al., 1986) even if successful breeding has occurred. With such losses inherent to the reproductive process, it is critical for veterinarians and managers to select the optimal time for breeding to maximize the potential for a successful pregnancy.

[0014] Current on-site methods for selecting the optimal time of breeding in the equine industry include the observation of behavioral interactions between the mare and stallion (teasing), rectal palpation and/or ultrasound to monitor ovarian follicular growth, as well as obligatory breed registry regulations. Teasing refers to the observation of the mare's behavior to the presence of a stallion. Distinct behavioral patterns are observed during each phase of the equine estrous cycle (Ginther, 1992). Palpation involves the insertion of the arm into the mare's rectum to manually determine ovarian follicular activity. Often, ultrasound viewing of ovarian follicular activity is also utilized. Accurate record of teasing and palpation/ultrasound data are essential for good breeding management.

[0015] Predicting optimal breeding time is also important because the nature of the breeding business adds constraints to successful conception. Most breeders do not keep stallions on their farms, and access to popular stallions requires scheduling and transportation of the mare to the stallion at a predetermined time (reserved booking date/time). This process adds substantial cost and financial risk and, therefore, increases the value of a tool that can predict ovulation. Furthermore, the arbitrary January 1 birth date employed by many breed registries requires that breeding and pregnancy occur as early in the breeding season as possible. Given a gestation length of 340 days, on average (Jeffcoat, 1972), early pregnancy results in birth as early in the following January as possible, creating a more mature, market valu-

able, horse for sale or training. According to the TBH Market Watch (1997), January foals were sold for 25% more compared to foals born in other months (i.e., February through July).

[0016] While predicting optimal breeding time is advantageous, few managers have the skill to accurately perform rectal palpation and even fewer have access to ultrasound. Veterinarians customarily charge managers for each farm visit and for each mare which they have to palpate and/or ultrasound. Moreover, traditional on-site breeding management practices (teasing, rectal palpation and/or ultrasound) cannot determine if a pre-ovulatory sized follicle will ovulate or regress. Accordingly, any technology which would allow the pre-selection of mares which require veterinary attention would be more efficient and economical.

[0017] The ovarian steroid hormone estradiol (E2) is a reliable indicator of the time of ovulation in mammals. During the breeding season, E2 is a critical hormone for normal follicular maturation, uterine endometrial development and ovulation in mares, as well as other mammals (Lipner, 1988). Furthermore, E2 exhibits a distinct secretory pattern during the estrous cycle during the breeding season. Mares are seasonal breeders with an annual reproductive cycle consisting of four phases: the breeding season (late spring to summer), the autumnal transition to Anestrus (late summer and fall), Anestrus (winter) and the vernal transition from Anestrus to the breeding season (late winter and spring) (Sharp, 1980). The breeding season is characterized by repeated estrous cycles, providing multiple, successive opportunities to become pregnant. Concentrations of E2 in blood are low (5 to 10 pg/ml) during the diestrus period; increase dramatically (30 to 70 pg/ml) in the 3 to 4 days preceding ovulation; peak 1 day prior to ovulation, on average; then decline to low diestrus concentrations (Pattison et al., 1974). Estrous cyclicity continues until either pregnancy results or declining day length initiates the transition into anestrus. Autumnal transition is not very well characterized. Briefly, a decline in hypothalamic-pituitary support, including GnRH (Strauss et al., 1979), LH and FSH secretion (Ginther, 1992), results in a progressive disruption in ovarian follicular growth and steroid hormone production. Anestrus begins when GnRH secretion reaches low, unvarying levels, LH and FSH secretion cease and ovarian follicular activity ceases (Ginther, 1992). Increasing day length in late winter initiates an increase in GnRH secretion and FSH secretion resulting in increased follicular growth (Ginther, 1992). An average of between 3 and 4 large, pre-ovulatory size follicles develop but fail to ovulate during this period (Tucker et al., 1993). These large, anovulatory follicles create considerable confusion as they are similar to ovulatory follicles in diameter, but they are unaccompanied by an increase in LH and they do not ovulate. Furthermore, these anovulatory follicles do not produce E2. LH secretion and E2 secretion do not recommence until immediately prior to the first ovulation of the year (the start of the breeding season) (Sharp et al., 1997).

[0018] Currently, veterinarians and breeding managers can determine E2 levels in mares only by submitting blood samples to a diagnostic laboratory. This process is costly and results are usually not available for 24 hours to one week, depending on the lab. Present commercial assay systems for E2 require several hours of incubation and expensive detection systems. Such assays utilize radioactive substances or

hazardous chemicals and complicated procedures, neither of which is compatible with on-site use. For example, U.S. Pat. No. 5,460,976 teaches an luminescence assay for measuring oestradiol in the blood sample of an equine using two antibodies, an antibody against human oestradiol and an antibody against human FSH. Unfortunately, in view of the extreme sensitivity of the immunologic components to both environmental condition and chemical environment, this method also does not lend itself to on-site use. Accordingly, the ability to rapidly and accurately measure E2, in the field, would greatly benefit veterinarians in assisting managers of equine breeding farms in making breeding management decisions.

BRIEF SUMMARY OF THE INVENTION

[0019] The subject invention concerns methods and materials for accurately assessing the presence or absence of an analyte of interest in a sample. The LBD is then used to detect the presence of a target analyte in a sample based on binding of the analyte by the LBD. In one embodiment, the subject invention involves utilizing a ligand binding domain (LBD) of a receptor to selectively capture a target analyte that is bound by the LBD. Accordingly, the methods of the invention can be used to detect any target analyte for which there is a receptor molecule having a ligand binding domain that specifically binds the target analyte. In one embodiment, the receptor molecule is a protein or polypeptide.

[0020] An exemplified embodiment of the present invention is directed to methods and materials for the rapid detection and quantitation of ovarian steroid hormone estradiol (E2) in the peripheral circulation of mammals, particularly horses. In this embodiment, the LBD utilized is a recombinantly-expressed polypeptide derived from the native equine estrogen receptor (eER) capable of specifically binding E2.

BRIEF DESCRIPTION OF THE DRAWINGS

[0021] FIG. 1 shows estradiol concentrations (pg/ml) in mares during two consecutive estrous cycles.

[0022] FIG. 2 shows estradiol concentrations (pg/ml) in mares during the vernal transition into the breeding season.

[0023] FIGS. 3A-3F show components and steps of a liquid phase assay of the present invention, wherein estradiol is the analyte of interest.

[0024] FIGS. 4A-4E show components and steps of a solid phase assay of the present invention, wherein estradiol is the analyte of interest.

[0025] FIGS. 5A-5C show components and steps of a lateral flow-based assay of the present invention, wherein estrogen is the analyte of interest.

BRIEF DESCRIPTION OF SEQUENCE SEQ ID NO:1 is an amino acid sequence of an equine estrogen receptor ligand binding domain according to the present invention.

DETAILED DESCRIPTION OF THE INVENTION

[0026] The subject invention concerns methods and materials for accurately assessing the presence or absence of an analyte of interest in a sample, particularly in physiological

samples. The methods of the invention utilize a ligand binding domain (LBD) of a receptor molecule that is capable of binding to a target analyte to selectively capture the analyte target specific for that LBD. In one embodiment, the receptor molecule is a protein or polypeptide. The ligand binding domain is allowed to react with a sample and the presence or amount of ligand (i.e., target analyte) bound by the LBD is determined. The subject invention includes ·within its scope any method or assay in which binding of an LBD to a target analyte can be used for detection or quantitation of the analyte.

[0027] The subject invention also concerns the ligand binding domain of the equine estrogen receptor. In an exemplified embodiment, the ligand binding domain has an amino acid sequence of SEQ ID NO:1, or a fragment or variant thereof that retains substantially the same ligand binding affinity as that associated with the polypeptide of SEQ ID NO:1. The subject invention also concerns polynucleotide sequences that encode a ligand binding domain of the present invention.

[0028] In one embodiment of the present methods, an LBD is attached to a solid support and contacted with a sample to be assayed for the presence or levels of a target analyte that the LBD specifically binds. After washing, a conjugate comprising target analyte conjugated with a detectable marker, such as an enzyme or radiolabel, is incubated with the LBD on the solid support. Levels of the conjugate bound to the LBD on the support are then determined.

[0029] In another embodiment, a sample to be assayed for the presence or level of a target analyte is contacted with a conjugate comprising an LBD for the analyte to be assayed conjugated to a detectable marker, such as an enzyme or a radiolabel. The sample is then contacted with target analyte immobilized to a support matrix, such as SEPHAROSE. After washing, the amount of the LBD-marker conjugate bound to the target analyte on the support matrix is determined.

[0030] In a further embodiment, a lateral flow based assay is provided in the present invention for the detection of a target analyte. A sample containing a target analyte of interest is added to an application well in a lateral flow device. The test sample flows toward a zone containing a solid support moiety that has been coated with a ligand-binding domain. When the sample front reaches this zone, the ligand-binding domain attached to the solid support moiety is released from the pad on which they are bound and then allowed to interact with target analyte that may be present in the test sample. Target analyte in the sample binds to the solid support bound LBD and flow continues toward a target analyte affinity matrix. All solid support moieties that possess "unoccupied" LBD are captured by the target analyte affinity matrix, whereas all solid support moieties having fully-occupied LBD will continue toward the capture zone where they are trapped. Results can be assessed visually by colorometric means with the intensity of color being directly proportional to target analyte concentration within the test sample.

[0031] The present invention provides a novel approach to analytical methods by utilizing a "capture protein" prepared by genetic engineering of a ligand binding domain of a naturally occurring receptor molecule for a target analyte.

The protein from which the LBD is derived has an amino acid sequence which is unique and binds the target analyte specifically. In an exemplified embodiment of an LBD of the present invention, a ligand binding domain portion of a receptor gene for E2 was produced by generating a cDNA fragment coding for the eER ligand binding domain (corresponding to amino acids 301-564 of the full-length receptor protein) by PCR utilizing the eER cDNA plasmid as template. PCR primers which flank the LBD of the eER were designed and synthesized by Gemini Biotech, Ltd. These primers also created XmaI restriction sites for subcloning of cDNA into a pBAD expression vector. Plasmid cDNA was isolated with QIAprep Spin DNA purification columns (Qiagen) and presence of the correct insert size was confirmed by restriction analysis. Following isolation of the eER-LBD subclone, the entire LBD coding region was sequenced to confirm no errors were incorporated by PCR amplification. The eER-LBD peptide was overexpressed in *E. coli* JM103 cells (available from ATCC, Rockville, Md.) transformed with the pBAD-23-eER-LBD expression vector. Purity of the protein was assessed by SDS-PAGE and Western blot and/or ligand blot analysis.

[0032] Use of a genetically engineered LBD as a "capture protein" has several advantages over existing systems. First, the ligand binding domain is specific to the hormone as it represents the naturally occurring tissue receptor mechanisms for recognizing a hormone. Second, use of the ligand binding domain as a "capture protein" is advantageous because the amino acid sequence can be altered by making point mutations in the oligonucleotide primers, and the properties of the ligand binding domain can then be adjusted to suit the needs of an assay system, or other systems requiring hormone-receptor recognition. Third, the in vitro expression of a ligand binding domain assures that a consistent, repeatable source of "capture protein" will be available. Fourth, the ligand binding domain can also be altered to accept organic linker arms for the purpose of attachment to solid phase, or to create attachment points for reporter molecules, such as enzymes, fluorescent molecules or other moities that can indicate the presence of the ligand binding domain having bound target analyte.

[0033] While a wide variety of analytes may be assessed utilizing the methods of the invention, the present disclosure exemplifies methods and materials of the invention for the detection of ovarian steroid hormone estradiol (E2). It should be understood, however, that the subject invention is not limited to the detection of estrogens and estradiol. Analytes which can be detected using the methods of the present invention include all those for which a ligand binding domain (LBD) can be derived.

[0034] Estradiol levels in mares increase significantly in blood just prior to the first ovulation of the year and just prior to each subsequent ovulation, as shown in FIG. 1. Traditional breeding management practices (teasing, rectal palpation and/or ultrasound) cannot determine if a pre-ovulatory size follicle will ovulate or regress. During vernal transition, E2 synthesis and secretion is very low or absent until the first preovulatory follicle of the year develops, where upon E2 concentrations in blood increase (30 to 70 pg/ml) dramatically (Davis et al., 1990), as shown in FIG. 2.

[0035] An exemplified embodiment of the present invention provides methods for persons such as veterinarians and

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breeding managers to determine if a follicle is a "vernal transition (non-ovulatory) follicle," or if the breeding season has begun (i.e., a fertile ovulation can be expected) and thereby make breeding management decisions accordingly. Components for the present methods are shown in **FIGS. 3A, 4A** and **5A**, and included a ligand-binding domain (LBD) of the equine estrogen receptor (eER). Sequence analysis of the LBD of the eER (nucleotide sequence of full-length eER gene: Genbank Assession #AF124093) shows 90% identity and 95% similarity to other mammalian ER-LBDs, as shown in Table 1.

TABLE 1

Comparison of the equine estrogen receptor nucleotide (AF124093) and deduced amino acid sequences to those of other species

| | | Full-length Estrogen Receptor | | | Ligand Binding Domain | |
Specie	GenBank Accession	Nucleotide Homology	Amino Acid Identity	Amino Acid Similarity	Amino Acid Identity	Amino Acid Similarity
Human	182192	89%	89%	93%	90.5%	94.9%
Mouse	193180	87%	87%	91%	90.5%	94.9%
Rat	56120	83%	86%	90%	90.2%	94.3%
Pig	587554	91%	91%	94%	90.5%	94.6%
Cow	1575521	ND	ND	ND	95.0%	96.0%
Sheep	1617201	89%	90%	94%	90.5%	94.9%
Chicken	63380	78%	76%	84%	85.5%	91.6%

[0036] Published data of all amino acids critical for E2 binding to the ER-LBD in other species (Ekena et al., 1996) are in identical sequence positions in the eER-LBD (Gly^{521}, His^{524}, Leu^{525}, Met^{528}). Therefore, the binding kinetics of the eER-LBD should not be different from data published for other species (K_d=0.1 nM)(Ekena et al., 1996). Such ER-LBD peptides can be expressed as individual peptides (e.g., residues 301 to 564 of eER) and maintain their specificity for E2 (Wrenn et al., 1993; Ekena et al., 1996).

[0037] Hence, it should be readily apparent by those of ordinary skill in the art that the inherent homology in the estrogen receptor nucleotide between mammalian species extends the utility of the subject invention to the diagnostic detection of E2 within mammalian species other than horses. For example, using either a modified or unmodified ER-LBD, the present invention is applicable to detection of ovulation in humans as well, particularly since preovulatory profiles of estrogen in women are even more robust than those in mares.

[0038] There are several alternative embodiments of the methods of the present invention that utilize a recombinantly-expressed polypeptide which contains a ligand binding domain (LBD) that can bind an analyte of interest. One embodiment concerns a liquid phase assay, the components for which are shown in **FIG. 3A**. A second embodiment concerns a solid phase assay, the components for which are shown in **FIG. 4A**. A third embodiment concerns a lateral flow assay, the components for which are shown in **FIG. 5A**. For the purpose of example, and as exemplified in the figures, the analyte of interest can be E2, in which case, a polypeptide containing the LBD of the estradiol receptor is used.

[0039] An exemplified embodiment of the methods of the present invention concerns a liquid phase-based assay for

equine E2 (FIGS. 3B-3F). In the first step, a sample, for example, blood from a mare to be tested is added to a titrated amount of an eER-LBD enzyme conjugate (hereafter referred to as "conjugate"). The conjugate is composed of a recombinantly-expressed LBD of equine estrogen receptor conjugated to a colorimetric enzyme, such as alkaline phosphatase or horseradish peroxidase. Conjugation of the eER-LBD with the colorimetric enzyme can be accomplished using standard methods. Preferably, the conjugate is titrated such that, when incubated with the sample, it will bind a maximum concentration of 30 pg/ml of E2, becoming saturated. The second step of the method consists of incubation to allow ligand binding. The mixture of conjugate and serum is then added to an estrogen affinity matrix. The estrogen affinity matrix functions as an estradiol affinity adsorbent, preferably consisting of a titrated amount of E2 immobilized upon SEPHAROSE (Amersham Pharmacia). The affinity matrix can be prepared by the linkage of estrogen with SEPHAROSE as described by Greene et al. (1980). Briefly, 17α-allylestradiol 3-acetate is prepared by the reaction of estrone with allylmagnesium chloride followed by acetylation of the phenolic group. The 17 α-allylestradiol 3-acetate is then converted to the side-chain epoxide by treatment with m-chloroperoxybenzoic acid and then reacted with reduced 2-hydroxy-3-mercapto-n-propyl-SEPHAROSE-6B. Unreacted sulfhydryl groups in the thiopropyl-SEPHAROSE will be blocked by treatment with iodoacetamide.

[0040] The affinity matrix is then washed and conjugate which has not bound to E2 within the sample will bind to the immobilized E2 on the affinity matrix and be "captured." However, conjugate which has bound E2 from the sample will flow through the matrix and, hence, not be captured. A color substrate can then be added to the estrogen affinity matrix, binding to the colorimetric enzyme component of any conjugate which has bound to the immobilized E2, as illustrated in **FIG. 3**. The intensity of the color will depend on the amount of complex "captured." Hence, color development occurs in a manner inversely proportional to analyte (E2) concentration within the sample. For example, if the mare's serum contains low concentrations of E2, an intense color will result, as most or all of the complexes will be captured on the matrix. However, if greater than 30 pg/ml of E2 are in the mare's serum, no color will result, as all the complexes will pass through the matrix without binding to the immobilized E2. While any number of color stages may be used, for unambiguous visualization it is preferable that

the conjugate be titrated in such a way that there is a three stage color possibility. For example, where the sample is mare's serum and the analyte of interest is E2, intense color indicates low E2, light color indicates between 5 and 25 pg/ml of E2 and no color indicates greater than 30 pg/ml E2.

[0041] While the color development is inversely proportional to the concentration of the analyte (17β-estradiol) in the test sample, to ensure color reactions that are visible to the eye, it should be understood that amplification of the colorimetric signal can be accomplished by using commercially available antibodies to horseradish peroxidase (HP) and alkaline phosphatase (AP). These antibodies can be biotinylated and amplification achieved with enzyme-labeled streptavidin. Alternatively, a sandwich assay which utilizes streptavidin and biotinylated enzyme can be incorporated to achieve maximal amplification.

[0042] The E2 can be immobilized onto the surface of the affinity support matrix by any method that affixes the E2 to the support in a substantially irreversible manner, such as where the E2 is covalently bound to the support matrix. Further, E2 can be attached or coupled to numerous other support matrices known in the art using standard methods. Suitable solid-phase support matrices can be composed of nitrocellulose, DEAE, glass, nylon, particulate silica, polystyrene, polyethylene, polyamides, polyacrylamides, polyvinyls, polypropylene, cellulose agarose, dextran or any other suitable material known in the art. The solid support matrix can be in the form of a vessel, a chamber, a dipstick, beads, particles, membranes, or other forms known in the art. Suitable membranes include those composed of nylon, nitrocellulose or polyvinylidenedifluoride (PVDF).

[0043] Because many color substrates are commercially available for each colorimetric enzyme and each has its own optimal conditions for color development, there are a variety of color substrates which can be used within the methods of · the present invention. Examples include, but are not limited to, p-Nitrophenyl Phosphate (PNPP; yellow), Fast Red (red) and 5-Bromo-4-Chloro-3'-Indolyphosphate (BCIP)/Nitro Blue Tetrazolium Chloride (NBT; black-purple) for the AP conjugates; and, 2,2'-Azinobis [3-ethylbenzothiazoline-6-sulfonic acid]-diammonium salt (ABTS; green), 3,3',5,5'-tetramethylbenzidine dihydrochloride (TMB; blue or yellow) and 3,3'-diaminobenzidine (DAB; brown) for the HP conjugates at the concentrations recommended by their supplier (Sigma Chemical Company and/or Pierce Chemical Company). The substrate which yields the most distinguishable changes in color in parallel to changes in analyte concentrations should be selected. However, stability of the substrate prior to the reaction, solubility of the product and sensitivity will all be considerations for substrate selection as well.

[0044] The methods of the present invention are also exemplified by a solid phase assay for detecting an analyte, such as E2 in an female equine (FIGS. 4B-4E). In this embodiment, a titrated concentration of eER-LBD is immobilized onto a support matrix, such as a nylon membrane, dipstick, coated vessel or filtration chamber. The solid support matrix can be composed of any of the materials described for the E2 affinity support matrix. As with the liquid phase embodiment, several concentrations of eER-LBD can be used. Preferably, the optimum titration will be that which is saturated by 30 pg/ml 17β-estradiol.

[0045] In the first step of the solid phase embodiment, immobilized (solid phase) eER-LBD is incubated at room temperature with the sample to allow for binding of eER-LBD with 17β-estradiol present in the sample. In the second step of the solid phase embodiment, the sample is then passed or drawn through or evacuated by vacuum filtration through the solid support matrix and the support matrix washed. Optionally, the wash solution can be a buffer that contains a blocking agent (e.g., gelatin or BSA) to reduce nonspecific binding. After the washing/blocking step, a titrated concentration of a 17β-estradiol-enzyme conjugate (hereafter referred to as the estrogen conjugate) is added to the membrane and incubated to allow interaction with the solid-phase eER-LBD that has not bound ligand. As with the liquid phase embodiment, the enzyme of the estrogen conjugate can be a colorimetric enzyme. After incubation, estrogen conjugate which is not bound by eER-LBD is removed and the solid support matrix can be washed. The solid support is then reacted with a substrate for the given enzyme conjugate as previously described in the liquid phase embodiment. The amount of colorimetric enzyme which remains on the solid support is inversely proportional to the amount of 17β-estradiol in the sample. It is preferable to titer the amount of solid-phase eER-LBD in such a way that a three-stage color development scheme will occur as described in the liquid phase embodiment. In addition, although the above described method utilizes a vacuum filtration manifold, the method can be easily adapted to other systems, such as a gravity flow system.

[0046] It should be appreciated that a ligand binding domain used in the methods of the present invention can be modified so as to adjust specificity and/or affinity to suit diagnostic needs. For example, a linker arm may be added to the LBD of the mammalian estrogen receptor for conjugation to plastic, enzymes, etc. In addition, the amino acid substituents of the LBD can be modified so as to alter the affinity of the LBD for its associated analyte. For example, mutations in the amino acid sequence of the LBD, such as amino acid substitutions, deletions and/or additions, are contemplated by the present invention.

[0047] Further, it should be understood by the ordinarily skilled artisan that the present invention may be further modified to resemble an "antibody sandwich" assay. For the purposes of this disclosure, the term "antibody sandwich" assay simply means an assay in which the analyte to be determined is "sandwiched" by an immunochemical reaction between a solid surface treated with a first antibody reactive with the analyte to be determined and the same or a different second antibody which has been coupled to an enzyme label. This "antibody variant" of the subject invention differs from traditional antibody sandwich assays in that the variant uses one antibody and the LBD of the subject invention, whereas traditional sandwich assays utilize two antibodies. For example, in the aforementioned liquid phase embodiment, a titrated amount of antibodies specific for the eER-LBD are immobilized upon the SEPHAROSE (Amersham Pharmacia) solid support, in lieu of 17β-estradiol. Alternatively, in the solid phase embodiment, an antibody-enzyme conjugate is added instead of the 17β-estradiol-enzyme conjugate. In this variant of the solid phase embodiment, the antibody is specific for the eER-LBD. In both of these antibody-variants of the liquid and solid phase embodiments, the amount of antibody used is titrated as previously described regarding the eER-LBD. Further,

monoclonal antibodies may be used in the assays, as disclosed in U.S. Pat. No. 4,376,110.

[0048] Additionally, U.S. Pat. No. 4,228,240 describes the stabilization of peroxidase containing compositions for use in enzyme immunoassay kits. U.S. Pat. No. 4,931,385 discloses an improved blocking solution which protects against nonspecific antibody binding and an improved antibody-enzyme conjugate which protects the antibody from loss of reactivity and immunological binding specificity even if the reagents had been subjected to hot, humid environmental conditions. Such reagents may be used in connection with the subject invention.

[0049] The methods of the invention can be used to detect any target analyte for which there is a protein that binds to the analyte and for which a ligand binding domain can be derived. Target analytes include analytes such as hormones, enzymes, lipoproteins, bacterial or viral antigens, immunoglobulins, lymphokines, cytokines, drugs, soluble cancer antigens, and the like. These analytes include various proteins such as protamines, histones, phosphorylated proteins, nucleoproteins, such as, for example, transcortin, erythropoietin, transferrin, various globulins, thyroxin-binding globulin, the immunoglobulins of various subclasses (IgA, IgG, IgD, IgE, and IgM), various complement factors, and blood clotting factors such as fibrinogen, Factor VIII, tissue thromboplastin, and thrombin. Further, the relationship of the binding pair (i.e., the target analyte and the protein from which the LBD is derived) is not limited. For example, the relationship may be one of enzyme-substrate, enzyme-inhibitor, enzyme-co-enzyme, etc. In addition to E2, other steroids including, but not limited to, progesterone and testosterone, and other hormones such as insulin, glucagon, relaxin, thyrotropin, somatotropin, luteinizing hormone, follicle-stimulating hormone, gastrin, bradykinin, vasopressin, and various releasing factors are suitable analytes. A wide range of antigenic polysaccharides can also be determined such as those from Chlamydia, *Neisseria gonorrheae, Pasteurella pestis, Shigella dysentereae,* and fungi such as Mycosporum and Aspergillus. Another major group comprises oligonucleotide sequences which react specifically with protein targets.

[0050] The test sample can be any material suspected of containing the analyte of interest. The sample can be derived from any source, such as physiological fluid, including blood, saliva, sweat, urine, milk, mucous, etc. The sample can be used as obtained directly from the source or following a pretreatment so as to modify its character. Pretreatment may involve separating plasma from blood, diluting viscous fluids, or the like. Methods of treatment can involve filtration, distillation, concentration, inactivation of interfering components, and the addition of reagents. For example, the test sample may be dissolved in or supplemented by a buffer to provide a suitable medium for the incubations of the invention. Further, in the case of the antibody variants of the subject invention, an additive may be included to facilitate immunologic reactions involving the antibody.

[0051] A further embodiment of the methods of the subject invention utilizes a modification to the lateral flow technique described in U.S. Pat. Nos. 4,943,522; 5,766,961; and 5,770, 460. In the first step of this embodiment, a biological sample containing the target analyte of interest (e.g., estrogen) is added to an application well in a lateral flow device. Lateral

flow is accomplished by incorporating a non-bibulous support, with inherent hydrophobic properties, which facilitates non-bibulous lateral flow of the test sample to various zones. The test sample flows toward a zone containing colored latex particles that have been coated with eER-LBD. The conjugation of the eER-LBD to latex particles can be by passive adsorption or via a covalent linkage. When the sample front reaches this zone, the colored latex particles are released from the pad from which they are impregnated and allowed to interact with the estrogen (analyte) present in the test sample. Estrogen in the sample binds to the latex bound eER-LBD and flow continues toward an estrogen affinity matrix. All latex particles that possess "unoccupied" eER-LBD are captured by the estrogen adsorbent, while all latex particles with fully-occupied eER-LBD will continue toward the capture zone where they are trapped in a very defined area. The capture zone can utilize a number of high affinity reactions including streptavidin-biotin or antibody-antigen interactions. Results are assessed visually with the number of captured beads, and thus intensity of color, being directly proportional to estrogen concentrations within the test sample (see **FIG. 5A**). Positive color development for physiological relevant concentrations of analyte is controlled by careful titration of the number of latex beads, the number of eER-LBD sites on each bead and/or the concentration of affinity adsorbent used.

[0052] It should be understood by those of ordinary skill in the art that various diagnostic devices usable in connection with other binding assays may be used in connection with the subject invention. For example, U.S. Pat. No. 4,361,537 and U.S. Pat. No. 4,855,240 disclose test devices comprising a highly absorbent material capable of transporting the test sample by capillarity. Further, certain devices rely on improved transverse flow through a filter in order to remove particulate and/or colored matter from the sample which may otherwise interfere with an accurate colorimetric reading, such as that disclosed in U.S. Pat. No. 4,623,461. It should also be appreciated by those of ordinary skill in the art that the present invention may be utilized in a device which uses a plurality of test elements, each for a different analyte, with all elements being supplied analyte from a single quantity of test sample, along different flow paths, as described in U.S. Pat. No. 4,323,536.

[0053] In addition, the eER-LBD of the present invention may be modified in order to detect and measure phytoestrogen within mammals, particularly agricultural animals such as horses and cows. Phytoestrogen is of concern to ranchers because phytoestrogen may accumulate in animals that eat plants with high amounts of phytoestrogen. Abnormal levels of phytoestrogen may then result in abnormal changes in the animal's estrous cycles.

[0054] All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety to the extent they are not inconsistent with the explicit teachings of this specification.

[0055] Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1

Cloning of the Equine Estrogen Receptor

[0056] The full-length equine estrogen receptor (eER; alpha-type; clone Kjm eER-13) was cloned from an estrous endometrial cDNA library (ZAP EXPRESS; Stratagene) using standard screening procedures (Sambrook et al., 1989). One microgram of eER cDNA plasmid was transformed into DH5ɑ competent cells and plated onto LB-agar. Two independent colonies were picked and selectively grown in 200 ml of Luria-Bertani (LB) culture broth with kanamycin (100 ug/ml). Equine ER cDNA plasmid was isolated from the growth cultures with the Plasmid Midi Kit (Qiagen). The cDNA insert (>>4000 bp) was sequenced in its entirety by the DNA sequencing core laboratory of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida. Nucleotide sequence comparisons were performed with the BLAST feature of the National Center for Biotechnology Information, as shown in Table 1. The eER nucleotide sequence was submitted to GenBank and assigned the accession number AF124093.

EXAMPLE 2

PCR Cloning of the eER Ligand Binding Domain

[0057] A cDNA fragment coding for the eER ligand binding domain (LBD; amino acids 301-564 based on the amino acid numbering of the full-length receptor sequence at Accession No. GI:4325290) was generated by PCR utilizing th eER cDNA plasmid as template. Oligonucleotides that flank the LBD of the estrogen receptor were designed and synthesized by Gemini Biotech, Ltd. and included XmaI sites at the 5' ends for future subcloning steps. Following 30 cycles of PCR (95° C. for 1 minute, 55° C. for 2 minute, 72° C. for 2 minutes), 10 μl of the PCR reaction was separated on a 1.8% agarose gel to confirm amplification of the correct product size (840 bp). One μl of the PCR reaction was ligated to pCR 2.1 cloning vector (Invitrogen) using the TA cloning principles. The ligation reaction (3 μl) was transformed into One Shot competent cells (Invitrogen) and plated onto LB-kanamycin agar plates that contained X-gal. Ten randomly selected recombinant (white) colonies were selected, inoculated into LB-ampicillin and grown overnight at 37° C. Plasmid cDNA was isolated with QIAprep Spin DNA purification columns (Qiagen) and presence of the correct insert size confirmed by restriction analysis. Following plasmid isolation, the eER-LBD clone was sequenced in its entirety by the DNA sequencing core laboratory of the Interdisciplinary Center for Biotechnology Research (ICBR) at the University of Florida to confirm no errors were incorporated by PCR amplification.

EXAMPLE 3

Subcloning into an Expression Vector

[0058] After confirming no errors were generated by PCR and that the coding sequence is in frame, the nucleotide sequence which codes for eER-LBD was released from the pCR 2.1 vector by digesting with XmaI and gel purifed. The eER-LBD coding sequence was then subcloned into a pBAD expression vector which had been modified to include a pectate lyase secretion signal (pBADPL; Gemini Biotech, U.S. Pat. No. 5,576,195). Furthermore, the pBADPL vector

added a 6X-histidine tag and a termination codon to the 3' end of the subclone. Five μl of the ligation reaction was transformed into the *E.coli* strain JM103 (ATCC) that had been rendered competent using $CaCl_2$ (Sambrook et al., 1989). After a 30 minute incubation on ice, cells were heat shocked for 1 minute at 42° C.; then grown for 1 hour at 37° C. in SOC media with shaking (250 rpm). Transformation reactions were plated onto LB-ampicillin plates and grown overnight at 37° C. Twenty single recombinant colonies were inoculated in LB medium containing 100 μg/ml ampicillin and grown overnight at 37° C. Plasmid DNA was isolated with QIAprep Spin DNA purification columns and orientation determined by restriction analysis and/or DNA sequencing. Two clones, one in the correct and one in the reverse orientation (negative control), was utilized for protein expression.

EXAMPLE 4

Recombinant Expression of the eER-LBD Peptide

[0059] Initially, pilot studies were conducted to determine optimal conditions for induction. Single recombinant colonies for each clone were selected from LB-ampicillin plates and inoculated into 2 ml of LB containing 100 μg/ml ampicillin. Cultures were grown at 37° C. with shaking (250 rpm) to an OD_{600}=1-2. Five 10-ml aliquots of LB-ampicillin were each inoculated with 100 μl of the overnight culture and grown at 37° C. with vigorous shaking to an OD_{600}=0.5 (mid-log phase). When an OD_{600}=0.5 was obtained, 1 ml of each culture was removed and saved for future analysis (Time 0 h). To the remaining 9 ml of each of the five respective growth cultures, 90 μl of a 10-fold serial dilution of 20% L-arabinose (0.002%-20%) was added such that the final arabinose concentrations ranged from 0.00002%-0.2%. Cultures were grown an additional 4 hours at 37° C. with shaking. One ml aliquots were removed (Time 4 h) and aliquots for both time points were centrifuged at maximum speed in a microfuge for 30 seconds. The supernatant (excreted protein) and the cell pellet (protein secreted into the periplasmic space) were added to Laemmli sample buffer, fractionated on a 12% gel by SDS-PAGE and stained with Coomassie blue. Gels were examined to determine the optimal arabinose concentration for induction as well as the proportion of the protein that is excreted into the medium. Once optimal conditions for maximum expression have been determined, expression can be scaled up accordingly depending upon the yields and the needs.

EXAMPLE 5

Affinity Purification of the Recombinant eER-LBD Peptide

[0060] The culture media can be concentrated with Centricon-plus 80 centrifugal filtration devices and dialyzed overnight against binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH=7.8) at 4° C. Total protein is determined with the BioRad protein assay. The eER-LBD protein is selected from the media by batch binding to ProBond resin (Invitrogen). An aliquot of the dialyzed media (equivalent to 5 mg of total protein) is brought to a total volume of 10 ml with binding buffer and divided into two 5-ml aliquots. One 5-ml aliquot is batch bound to 5 ml of ProBond resin with gentle rocking for 10 minutes at room temperature. The resin is settled by centrifugation at 800×g

and the supernatant decanted. This is repeated with the second 5-ml aliquot. The resin is then washed three times with a native wash buffer (20 mM sodium phosphate, 500 mM sodium chloride, pH=6.0) by resuspending the resin in 10 ml of the wash buffer, rocking for 2 minutes and separating by centrifugation at 800×g. After the final wash, the resin is transferred to a column. The eER-LBD protein is eluted from the resin by consecutively adding 10 ml of each of four imidazole buffers (50 mM, 100 mM, 250 mM, 500 mM), collecting 1 ml fractions and monitoring the OD_{280} of each fraction. Fractions with peak absorbance are pooled and purity is assessed by SDS-PAGE. Protein is quantified with the BioRad protein assay after dialysis to remove the imidazole. Purity of the protein is assessed by SDS-PAGE and Western blot analysis.

EXAMPLE 6

Scatchard Analysis for Estrogen Binding to
eER-LBD

[0061] After expression and purification, the equilibrium binding affinity of eER-LBD for [^{3}H]17β-estradiol can be determined by saturation analysis. Receptor preparations are diluted in TEDG buffer (10 mM Tris, 1.5 mM EDTA, 1 mM dithiothreitol, 10% vol/vol glycerol; pH 7.8). Dilutions (1-2 nM) of the receptor preparation are then incubated at 4 C overnight with increasing concentrations (0.2-20 nM) of [^{3}H]17β-estradiol. Nonspecific binding is determined in the presence of a 200-fold excess of unlabeled 17β-estradiol. Free ligand is separated from bound ligand by addition of an equal volume of dextran-coated charcoal slurry (1% charcoal, 0.01% dextran in TEDG). After a 10 minute incubation on ice, the charcoal is pelleted by centrifugation for 5 minutes at 14,000 rpm in a microfuge. The supernatent is carefully decanted and a portion used to quantitate [^{3}H]17β-estradiol binding by liquid scintillation counting using SCINTIVERSE cocktail. All data is then transformed by the method of Scatchard (1949) and an equilibrium dissociation constant (K_d) determined for the eER-LBD. The calculated K_d value can compare to those of the ER-LBD expressed in other species and by other expression systems.

EXAMPLE 7

Dissociation Kinetics of Estradiol from eER-LBD

[0062] The dissociation of [^{3}H]17β-estradiol from the eER-LBD peptide can be measured by the exchange of [^{3}H]17β-estradiol with an excess of unlabeled 17β-estradiol. The eER-LBD peptide (1-2 nM) is incubated with saturating (10 nM) concentrations of [^{3}H]17β-estradiol at 4° C. overnight. Samples are then pre-incubated in a 29° C. waterbath for 30 minutes prior to the addition of a 1000-fold excess of unlabeled 17β-estradiol. Dissociation is allowed to progress at 29° C. and aliquots are removed every 30 minutes for 8 hours. Nonspecific binding is determined by performing the overnight incubation in the presence of a 200-fold excess of unlabeled 17β-estradiol. Dissociation-rate experiments are terminated by DEAE filtration as described by Salomonsson et al. (1993). Briefly, DEAE paper discs (DE 81, Whatman International Ltd) are put into a filtration manifold and the sample is applied to the dry disc. A 2 minute incubation is performed prior to vacuum filtration to allow the eER-LBD-estrogen complex to bind to the filter disc. After vacuum is applied, the discs are washed with 10 volumes of ice-cold

Tris buffer (pH 7.8) and transferred to scintillation vials. Radioactivity is allowed to dissolve in the scintillation cocktail for 4 hours before quantitation. Data is then presented as the amount of [^{3}H]17β-estradiol displaced (percent of the initial binding at time 0) as a function of time.

EXAMPLE 8

Stability of the Unoccupied eER-LBD

[0063] The stability of the eER-LBD can be determined by measuring the amount of specific [3H]17β-estradiol binding observed after increasing incubation times at 0° C. or 25° C. After a pre-incubation at either 0° C. or 25° C. for 2-24 hours, specific binding is determined as described above. Results are presented as the percentage specific binding relative to the initial binding capacity as a function of time.

EXAMPLE 9

Conjugation of eER-LBD with Colorimetric
Enzymes

[0064] The eER-LBD peptide can be conjugated with alkaline phosphatase (AP) or horseradish peroxidase (HP) using preactivated enzymes and the EZ-Link conjugation kits according to the manufacturer's recommendations (Pierce Chemical Company). For AP conjugation, maleimide activated AP is reacted with free sulfhydryl (—SH) groups present in the eER-LBD peptide to form a stable thiol ether linkage (Ishikawa et al., 1983). The AP-eER-LBD conjugate is purified by gel filtration chromatography, adjusted to a protein concentration equivalent to 1 nM eER-LBD and utilized for binding studies. For HP conjugation, periodate activated HP is reacted with amine (—NH$_2$) residues present in the eER-LBD peptide to form a covalent amide bond (Imagawa et al., 1982). After conjugation, the linkage is reduced and the activated HP will be quenched with ethanolamine. The HP-eER-LBD conjugate will be purified on a desalting column, adjusted to a protein concentration equivalent to 1 nM eER-LBD.

EXAMPLE 10

Conjugation of 17β-estradiol with Colorimetric
Enzymes

[0065] The conjugation of 17β-estradiol with either HP or AP can be accomplished a number of different ways including the mixed anhydride method (Munro et al., 1984), the carbodiimide method and the modified carbodiimide method, which uses an activated estradiol ester prepared with N-hydroxysuccinimide (Munro et al., 1988). Preferably, the mixed anhydrid method is utilized. Briefly, a derivative (hemisuccinate or carboxymethyloxime) of 17β-estradiol (Steraloids) and sec-butylchlorocarbonate are dissolved in N,N-dimethylformamide at 0° C. N-methylmorpholine is added to remove hydrochloric acid and form the mixed anhydride. In a separate reaction, enzyme (AP or HP) is dissolved in water and N,N-dimethylformamide is added. The steroid solution that contains the mixed anhydride is gradually added to the enzyme solution at 0° C. The reaction mixture is stirred for 60 minutes at -20° C. then an additional 120 minutes at 0° C. After the incubation, sodium bicarbonate is added and the reaction mixture is dialyzed overnight against distilled water at 4° C. The dialysate is

passed over a Sephadex G-25 column and the 17β-estradiol-enzyme conjugate is aliquoted and stored at –20° C.

EXAMPLE 11

Conjugation of Domain F of eER-LBD with Colorimetric Enzymes

[0066] A specific conjugation site can be added to the carboxy-terminus of the expressed eER-LBD peptide (Pierce). This is not as desirable, however, since conjugation is 1:1 (enzyme:eER-LBD). Since the expressed peptide can contain not only the LBD (domain E) of the ER but also domain F, tagging the carboxy-terminus does not impede ligand-receptor interactions since crystallographic studies have shown this region is not an integral part of the binding pocket of the estrogen receptor (Tanenbaum et al., 1998).

EXAMPLE 12

Coating Latex Particles with eER-LBD by Passive Adsorption

[0067] The recombinant eER-LBD is coated onto surfactant-free polystyrene latex particles (Interfacial Dynamics Corp.) via physical adsorption. Most commercially available latex particles are hydrophobic and, thus, will adsorb proteins strongly and irreversibly via the hydrophobic domains in the proteins. Briefly, 2.5 ml or 1.25 ml of a 4% or 8% solids, respectively, suspension of colored latex particles (e.g. those with sulfate or carboxyl surface functional groups) is diluted to 10 ml with 25 mM 2-[N-Morpholino] ethanesulfonic acid, pH=6.0 (MES). The suspension is then centrifuged at 3,000×g for 20 minutes to sediment the latex particles. The supernatant is decanted and the latex particles re-dispersed in an additional 10 ml of MES. This mixture is centrifuged at 3,000×g to sediment the latex particles; and, the resultant supernatant is discarded and the pellet resuspended in 5 ml of MES to yield a latex suspension of approximately 2% solids. An equal volume of the 2% latex suspension is added to a 1 mg eER-LBD/ml MES solution (assuming latex particle size is 1 μM). The concentration of eER-LBD can be scaled up or down for smaller and larger particle sizes, respectively. The latex particle/eER-LBD mixture is incubated overnight with gentle mixing at room temperature. The unbound eER-LBD is separated from the eER-LBD-labeled latex particles by centrifugation. The supernatant is saved and a protein determination is performed using the Micro BCA Protein Determination Kit (Pierce). The pellet is resuspended in 10 ml of phosphate-buffered saline, pH=7.2 (PBS) and centrifuged to sediment the particles. This washing step is repeated twice for a total of three washes. The final latex pellet is suspended in the original coupling volume (final concentration of 1.0% solids) of PBS amended with 0.1% glycine and 0.1% sodium azide (storage buffer) and stored at 4° C. Glycine will cover any reactive sites on the microsphere surface not occupied by eER-LBD and will ultimately reduce nonspecific binding. The amount of eER-LBD coupled to the latex particles is determined by subtracting the residual protein measured in the supernatant from the original amount added. The binding properties of the eER-LBD-labeled latex particles are assessed as previously described except that bound is separated from free via centrifugation.

EXAMPLE 13

Coating Latex Particles with eER-LBD by Covalent Coupling

[0068] Although passive adsorption is the preferred method of coating latex particles, primary amino groups of protein molecules can also be covalently coupled to carboxyl functional groups on the latex particles using a water soluble carbodiimide such as 1-ethyl-3-(3-dimethyl amino propyl)carbodiimide-HCl (EDAC). Latex suspensions are brought to 2% solids as described above for passive adsorption. This procedure will work with either carboxyl latex or carboxylate-modified latex (CML). To the latex, 2 ml of EDAC in MES (50 mg/ml) and 3 ml of eER-LBD (approximately 5 mg of protein) is added. The latex/protein mixture is incubated at room temperature for 3-4 hours on a rocking platform. Unbound eER-LBD is removed by centrifugation and the supernatant retained for protein determination. The eER-LBD-labeled latex particles is washed 3 times in PBS and resuspended to 1% solids in storage buffer and stored at 4° C. until used to characterize binding properties as previously described except that bound is separated from free via centrifugation.

[0069] It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application and the scope of the appended claims.

References

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[0071] U.S. Pat. No. 5,576,195

[0072] U.S. Pat. No. 4,376,110

[0073] U.S. Pat. No. 4,228,240

[0074] U.S. Pat. No. 4,931,385

[0075] U.S. Pat. No. 4,943,522

[0076] U.S. Pat. No. 5,766,961

[0077] U.S. Pat. No. 5,770,460

[0078] U.S. Pat. No. 4,361,537

[0079] U.S. Pat. No. 4,855,240

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Ala Thr Arg Pro Phe Asn Glu Ala Ser Met Met Gly Leu Leu Thr Asn
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Leu Ala Asp Arg Glu Leu Val His Met Ile Asn Trp Ala Lys Arg Val
    50                  55                  60

Pro Gly Phe Val Asp Leu Ser Leu His Asp Gln Val His Leu Leu Glu
65                  70                  75                  80

Cys Ala Trp Leu Glu Ile Leu Met Ile Gly Leu Val Trp Arg Ser Met
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Glu His Pro Gly Lys Leu Leu Phe Ala Pro Asn Leu Leu Leu Asp Arg
            100                 105                 110

Asn Gln Gly Lys Cys Val Glu Gly Met Val Glu Ile Phe Asp Met Leu
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Phe Val Cys Leu Lys Ser Ile Ile Leu Leu Asn Ser Gly Val Tyr Thr
145                 150                 155                 160

Phe Leu Ser Ser Thr Leu Lys Ser Leu Glu Glu Lys Asp His Ile His
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Arg Val Leu Asp Lys Met Thr Asp Thr Leu Ile His Leu Met Ala Lys
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Ile Leu Ser His Ile Arg His Met Ser Asn Lys Gly Met Glu His Leu
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Tyr Ser Met Lys Cys Lys Asn Val Val Pro Leu Tyr Asp Leu Leu Leu
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Glu Met Leu Asp Ala His Arg Leu His Ala Pro Ala Asn His Gly Gly
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Ala Pro Met Glu Glu Thr Asn Gln
                260
```

We claim:

1. A method for detecting a target analyte in a sample, said method comprising:

(a) contacting a sample with a ligand binding domain of a molecule that is capable of selectively binding to said target analyte; and

(b) determining whether said target analyte has been bound by said ligand binding domain.

2. The method according to claim 1, wherein said molecule is a protein or polypeptide.

3. The method according to claim 1, wherein said ligand binding domain is from a mammalian estrogen receptor.

4. The method according to claim 3, wherein said mammalian estrogen receptor is equine estrogen receptor.

5. The method according to claim 1, wherein said target analyte is a mammalian steroid.

6. The method according to claim 5, wherein said mammalian steroid is selected from the group consisting of estradiol, progesterone, and testosterone.

7. The method according to claim 6, wherein said estradiol is equine estradiol.

8. The method according to claim 1, wherein said target analyte is selected from the group consisting of protein, hormone, antigen, enzyme, drugs, environmental pollutant, lipoprotein, polysaccharide, immunoglobulin, lymphokine, cytokine, soluble cancer antigen, and oligonucleotides that bind specifically with a protein.

9. The method according to claim 8, wherein said protein is selected from the group consisting of protamine, histone, phosphorylated protein, nucleoprotein, globulin, complement factors, and blood clotting factors.

10. The method according to claim 9, wherein said blood clotting factor is selected from the group consisting of fibrinogen, Factor VIII, tissue thromboplastin, and thrombin.

11. The method according to claim 9, wherein said nucleoprotein is selected from the group consisting of transcortin, erthropoietin, and transferrin.

12. The method according to claim 8, wherein said hormone is selected from the group consisting of insulin, glucagon, relaxin, thyrotropin, somatotropin, luteinizing hormone, follicle-stimulating hormone, gastrin, bradykinin, vasopressin, steroid hormones which bind to an estrogen receptor, and releasing factors.

13. The method according to claim 8, wherein said polysaccharide is an antigenic polysaccharide from Chlamydia, *Neisseria gonorrheae, Pasteurella pestis, Shigella dysentereae,* Mycosporum and Aspergillus.

14. The method according to claim 1, wherein said sample is a physiological fluid.

15. The method according to claim 14, wherein said physiological fluid is selected from the group consisting of blood, serum, saliva, sweat, urine, milk and mucous secretions.

16. The method according to claim 1, wherein said ligand binding domain is attached to a solid support.

17. The method according to claim 16, further comprising the following steps after step (a):

washing said solid support to remove unbound materials and contacting said solid support with a conjugate comprising said target analyte conjugated to a detectable marker.

18. The method according to claim 16, wherein said solid support is selected from the group consisting of nitrocellulose, DEAE, glass, nylon, particulate silica, polystyrene, polyethylene, polyamides, polyacrylamides, polyvinyls, polypropylene, cellulose agarose, dextran, and sepharose.

19. The method according to claim 17, wherein said detectable marker is selected from the group consisting of enzyme, radiolabel, and fluorescent molecules.

20. The method according to claim 1, wherein said ligand binding domain is conjugated to a detectable marker.

21. The method according to claim 20, further comprising the following steps after step (a):

contacting the mixture of said sample and said ligand binding domain with said target analyte immobilized to a solid support and washing said solid support to remove unbound materials.

22. The method according to claim 21, wherein said solid support is selected from the group consisting of nitrocellulose, DEAE, glass, nylon, particulate silica, polystyrene, polyethylene, polyamides, polyacrylamides, polyvinyls, polypropylene, cellulose agarose, dextran, and sepharose.

23. The method according to claim 20, wherein said detectable marker is selected from the group consisting of enzyme, radiolabel, and fluorescent molecules.

24. The method according to claim 16, wherein said method is performed using lateral flow along a non-bibulous support having hydrophobic properties, said non-bibulous support comprising:

(1) a first zone coated with said ligand binding domain attached to said solid support;

(2) a second zone coated with said target analyte; and

(3) a third zone coated with a capture agent capable of capturing said ligand binding domain attached to said solid support,

wherein said sample contacts said first zone and releases said ligand binding domain attached to said solid support which flows to said second zone, said second zone binding any ligand binding domain not bound by target analyte in said sample, wherein unbound ligand binding domain attached to said solid support flows to said third zone and is captured by said capture agent.

25. The method according to claim 24, wherein said solid support is conjugated with a moiety that is specifically bound by said capture agent.

26. The method according to claim 25, wherein said moiety is selected from the group consisting of avidin, biotin, antibody, and antigen, and wherein said capture agent is the corresponding binding partner of said moiety.

27. The method according to claim 24, wherein said solid support comprises a colored latex particle.

28. A ligand binding domain of a receptor molecule, or a fragment of said ligand binding domain, that can bind to a target analyte specific for said ligand binding domain.

29. The ligand binding domain according to claim 28, wherein said receptor molecule is a protein or polypeptide.

30. The ligand binding domain according to claim 28, wherein said ligand binding domain is from a mammalian estrogen receptor.

31. The ligand binding domain according to claim 30, wherein said mammalian estrogen receptor is an equine estrogen receptor.

32. The ligand binding domain according to claim 31, wherein said ligand binding domain of said equine estrogen receptor has the amino acid sequence of SEQ ID NO:1, or a fragment thereof.

33. A polynucleotide comprising a coding sequence, wherein said coding sequence encodes a ligand binding domain of a receptor molecule, or a fragment of said ligand binding domain, that can bind to a target analyte specific for said ligand binding domain.

34. The polynucleotide according to claim 33, wherein said ligand binding domain is from a mammalian estrogen receptor.

35. The polynucleotide according to claim 33, wherein said mammalian estrogen receptor is an equine estrogen receptor.

36. The polynucleotide according to claim 35, wherein said ligand binding domain of said equine estrogen receptor has the amino acid sequence of SEQ ID NO:1, or a fragment thereof.

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(54) **FOLLICLE STIMULATING HORMONES**

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(57) **ABSTRACT**

Heterodimeric polypeptide conjugates exhibiting FSH activity, comprising a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein at least one of the FSH-α and FSH-β subunits differs from the corresponding wildtype subunit in that at least one amino acid residue acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed, and having at least one non-polypeptide moiety bound to an attachment group of at least one of said subunits are provided. Preferably, at least one attachment group, e.g., an N- or O-glycosylation site or an attachment site for a polymer molecule such as polyethylene glycol, has been introduced, e.g., at an N-terminal. The polypeptide conjugates exhibit improved properties, in particular an increased half-life, compared to human FSH.

FIGURE 1

Sequence alignment of human FSH to the structural part of two published structures of Human Chorionic Gonadotropin ("1HRP" and "1HCN"). The "/" indicates the chain break between the alpha and the beta chain.

```
FSH     -QDCPECTLQ ENPFFSQPGA PILQCMGCCF SRAYPTPLRS KKTMLVQKNV

1HRP    TQDCPECTLQ ENPFFSQPGA PILQCMGCCF SRAYPTPLRS KKTMLVQKNV

1HCN    -QDCPECTLQ ENPFFSQPGA PILQCMGCCF SRAYPTPLRS KKTMLVQKNV


FSH     TSESTCCVAK SYNRVTVMGG FKVENHTACH CSTCYY/--- --NSCELTNI

1HRP    TSESTCCVAK SYNRVTVMGG FKVENHTACH CSTCYY/KEP LRPRCRPINA

1HCN    TSESTCCVAK SYNRVTVMGG FKVENHTACH CSTCYY/KEP LRPRCRPINA


FSH     TIAIEKEECR FCISINTTWC AGYCYTRDLV YKDPARPKIQ KTCTFKELVY

1HRP    TLAVEKEGCP VCITVNTTIC AGYCPTMTRV LQGVLPALPQ VVCNYRDVRF

1HCN    TLAVEKEGCP VCITVNTTIC AGYCPTMTRV LQGVLPALPQ VVCNYRDVRF


FSH     ETVRVPGCAH HADSLYTYPV ATQCHCGKCD SDSTDCTVRG LGPSYCSFGE

1HRP    ESIRLPGCPR GVNPVVSYAV ALSCQCALCR RSTTDCGGPK DHPLTCD...

1HCN    ESIRLPGCPR GVNPVVSYAV ALSCQCALCR RSTTDCGGPK DHPLTCD...


FSH     MKE

1HRP    ...

1HCN    ...
```

FOLLICLE STIMULATING HORMONES

CROSS-REFERENCES TO RELATED APPLICATIONS

[0001] This application claims priority to and benefit of the following international and United States Patent Applications: Danish Patent Application PA 2000 00220, filed Feb. 11, 2000; U.S. Patent Provisional Application No. 60/184,035, filed Feb. 22, 2000; Danish Patent Application PA 2000 01092, filed Jul. 14, 2000; and U.S. Provisional Application No. 60/225,558, filed Aug. 16, 2000, the specifications of which are incorporated herein in their entirety for all purposes.

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FIELD OF THE INVENTION

[0003] The present invention relates to new polypeptides and polypeptide conjugates exhibiting follicle stimulating hormone (FSH) activity, to methods for preparing such polypeptides and conjugates, and to the use of such polypeptides and conjugates in therapy, in particular in the treatment of infertility.

BACKGROUND OF THE INVENTION

[0004] Follicle Stimulating Hormone (FSH) is a dimeric hormone consisting of an α subunit and a β subunit. The α subunit is common to the glycoprotein hormone family, which in addition to FSH includes chorionic gonadotropin (CG), thyroid stimulating hormone (TSH), and luteinizing hormone (LH). The β subunit is specific to FSH. The human wildtype α subunit is a 92 amino acid glycoprotein, the amino acid sequence of which is shown in SEQ ID NO:2. Said subunit is referred to herein as hFSH-α. The human wildtype β subunit is a 111 amino acid glycoprotein that has the amino acid shown in SEQ ID NO:4. This subunit is referred to herein as hFSH-β.

[0005] Human FSH (hFSH) has been isolated from pituitary glands and from post-menopausal urine (EP 322 438) and has been produced recombinantly in mammalian cells (U.S. Pat Nos. 5,639,640, 5,156,957, 4,923,805, 4,840,896, 5,767,251, EP 211,894 and EP 521,586). The latter references also disclose the hFSH-β gene. U.S. Pat. No. 5,405,945 discloses a modified human α subunit gene comprising only one intron.

[0006] U.S. Pat. Nos. 4,589,402 and 4,845,077 disclose purified hFSH which is free of LH and the use thereof for in vitro fertilization. EP 322 438 discloses a protein with at least 6200 U/mg FSH activity which is substantially free of LH activity, and wherein the FSH α subunit and β subunit, respectively, can be wildtype or specified truncated forms thereof.

[0007] Liu et al., *J Biol Chem* 1993, 15;268(2):21613-7, Grossmann et al., *Mol Endocrinol* 1996 10(6): 769-79, Roth and Dias (*Mol Cell Endocribol* 1995 1; 109(2): 143-9, Valove et al., *Endocrinology* 1994; 135(6):2657-61 ,Yoo et al., *J Biol Chem* 1993 25; 268(18): 13034-42), U.S. Pat. No. 5,508,261 and Chappel et al., 1998, *Human Reproduction*, 13(3): 18-35 disclose various structure-function relationship studies and identify amino acid residues involved in receptor binding and activation and in dimerization of FSH.

[0008] It has been found that glycosylation of FSH-α and FSH-β is essential for receptor signal transduction. hFSH-α comprises two N-glycosylation sites at the asparagines located at position 52 and 78, whereas hFSH-β comprises two N-glycosylation sites at the asparagines located at positions 7 and 24. The importance of the various N-glycosylation sites for the binding and signal-transducing activities of FSH are discussed, inter alia, by Valove et al., *Endocrinology* 1994; 135(6):2657-61 and Flack et al., *J Biol Chem* 1994 13;269(19):14015-20.

[0009] Galway et al., *Endocrinology* 1990; 127(1):93-100 demonstrate that FSH variants produced in a N-acetylglucosamine transferase-I CHO cell line or a CHO cell line defective in sialic acid transport are as active as FSH secreted by wildtype cells or purified pituitary FSH in vitro, but lacked in vivo activity, presumably due to rapid clearance of the inadequately glycosylated variants in serum. D'Antonio et al., *Human Reprod* 1999; 14(5):1160-7 describe various FSH isoforms circulating in the blood stream. The isoforms have identical amino acid sequences, but differ in their extent of post-translational modification. It was found that the less acidic isoform group had a faster in vivo clearance as compared with the acidic isoform group, possibly due to differences in the sialic acid content between the isoforms.

[0010] U.S. Pat. No. 5,087,615 discloses a method for stimulating follicle development and ovulation in a female patient by administering FSH to said patient during the follicular phase of the ovulatory cycle, the improvement comprising initially adminstering a first FSH isoform having a relatively long plasma half-life and subsequently administering a second FSH isoform having a shorter plasma half-life.

[0011] Bishop et al. *Endocrinology* 1995; 136(6):2635-40 conclude that circulatory half-life appears to be the primary determinant of in vivo activity.

[0012] Attempts have been made to prolong the serum half-life of FSH. U.S. Pat. Nos. 5,338,835 and 5,585,345 disclose a modified FSH-β subunit extended at the C-terminal Glu with the carboxy terminal portion (CTP) region of hCG (the region consisting of the amino acid sequence which occurs from positions 112-118 to 145, and comprising four O-linked glycosylation sites located at positions 121, 127, 132 and 138). The resulting modified subunit is stated to have the biological activity of native FSH, but a prolonged circulating half-life. U.S. Pat. No. 5,405,945 discloses that the carboxy terminal portion of the CG β subunit or a variant thereof has significant effects on the clearance of CG, FSH, and LH.

[0013] U.S. Pat. No. 5,883,073 discloses single-chain proteins comprised of two α-subunits with agonist or antagonist activity for CG, TSH, LH and FSH.

[0014] U.S. Pat. No. 5,508,261 discloses heterodimeric polypeptides having binding affinity to LH and FSH receptors comprising a glycoprotein hormone α subunit and a non-naturally occurring β subunit polypeptide, wherein the β subunit polypeptide is a chain of amino acids comprising four joined subsequences, each of which is selected from a list of specific sequences.

[0015] U.S. Pat. No. 5,567,422 and WO 98/32466 mention FSH among a vast number of other therapeutic proteins that can be PEGylated.

[0016] Currently, FSH is used therapeutically to stimulate the growth and maturation of ovarian follicles in infertile women. In particular, FSH is used in connection with in vitro fertilization as well as for the treatment of anovulatory women, with anovulatory syndrome or luteal phase deficiency. However, one problem encountered in current FSH treatment is the fairly short in vivo half-life of FSH requiring frequent, usually daily administration of the product. The frequent administration is very inconvenient for the patient and results in high fluctuations of FSH activity in the blood stream, which can cause inadequate maturation of the follicles.

[0017] Therefore, a clinical need exists for a product which provides part or all of the therapeutically relevant effects of FSH, and which can be administered at less frequent intervals as compared to currently available FSH product, and which preferably provides a more stable level of circulating FSH activity as compared to that obtainable by current treatment. The present invention provides such products as well as the means of making such products.

SUMMARY OF THE INVENTION

[0018] The present invention relates to polypeptide conjugates exhibiting FSH activity and methods for their preparation and their use in medical treatment.

[0019] Accordingly, in a first aspect, the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising i) a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein at least one of said FSH-α and FSH-β subunits differs from the corresponding wildtype subunit in that at least one amino acid residue acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed, and ii) at least one non-polypeptide moiety bound to an attachment group of at least one of said subunits.

[0020] In another aspect, the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising i) a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein the amino acid sequence of at least one of said FSH-α and FSH-β subunits differs from that of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced, and ii) at least one oligosaccharide moiety bound to an N-glycosylation site of at least one of said subunits.

[0021] In a further aspect, the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a dimeric polypeptide comprising FSH-α and FSH-β subunits, wherein at least one of said FSH-α and FSH-β subunits comprises, relative to the corresponding wildtype subunit, at least one introduced N- or O-glycosylation site at the N-terminal thereof, said at least one introduced glycosylation site being glycosylated.

[0022] In the above aspects, the corresponding wildtype subunits are preferably hFSH-α and hFSH-β, respectively.

[0023] Another aspect of the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein at least one of said FSH-α and FSH-β subunits comprises a polymer molecule bound to the N-terminal thereof.

[0024] In a further aspect, the invention relates to modified FSH-α and modified FSH-β polypeptides that can be used as intermediate products for the preparation of a conjugate with a polymer molecule.

[0025] In still further aspects, the invention relates to methods for preparing a conjugate or a polypeptide of the invention, including nucleotide sequences and expression vectors encoding a polypeptide or a conjugate of the invention.

[0026] In yet other aspects, the invention relates to a composition comprising a conjugate or polypeptide of the invention and methods of treating a mammal with such composition. In particular, the polypeptide, conjugate or composition of the invention can be used to treat infertility.

BRIEF DESCRIPTION OF THE FIGURES

[0027] FIG. 1 shows a sequence alignment of human FSH to the structural part of two published structures of human chorionic gonadotropin.

DETAILED DISCUSSION

[0028] Definitions

[0029] In the context of the present application and invention the following definitions apply:

[0030] The term "conjugate" is intended to indicate a heterogeneous molecule formed by the covalent attachment of one or more polypeptides to one or more non-polypeptide moieties such as polymer molecules, oligosaccharide moieties, lipophilic compounds, carbohydrate moieties or organic derivatizing agents. The term covalent attachment means that the polypeptide and the non-polypeptide moiety are either directly covalently joined to one another, or else are indirectly covalently joined to one another through an intervening moiety or moieties, such as a bridge, spacer, or linkage moiety or moieties. Preferably, the conjugate is soluble at relevant concentrations and conditions, i.e., soluble in physiological fluids such as blood. The term "non-conjugated polypeptide" can be used about the polypeptide part of the conjugate.

[0031] The term "polypeptide" can be used interchangeably herein with the term "protein." Further, the terms "polypeptide" and "protein" are generally used herein for the sake of simplicity to refer to the heterodimeric FSH polypeptides/proteins and conjugates of the invention, even though these proteins strictly speaking comprise a dimer of the α and β polypeptide subunits. The individual subunits are referred to herein as FSH-α and FSH-β, respectively, so that it is clear from the context whether reference is made to the dimeric hormone or to one of the subunits.

[0032] The "polymer molecule" is a molecule formed by covalent linkage of two or more monomers, wherein none of the monomers is an amino acid residue, except where the polymer is human albumin or another abundant plasma protein. The term "polymer" can be used interchangeably with the term "polymer molecule." The term is intended to cover carbohydrate molecules attached by in vitro glycosylation. Carbohydrate molecules attached by in vivo glycolsylation, such as N- or O-glycosylation (as further described below) are referred to herein as "an oligosaccharide moiety." Except where the number of polymer molecules is expressly indicated, every reference to "a polymer," "a polymer molecule," "the polymer" or "the polymer molecule" contained in polypeptide of the invention or otherwise used in the present invention shall be a reference to one or more polymer molecule(s).

[0033] The term "attachment group" is intended to indicate an amino acid residue group of the polypeptide capable of coupling to the relevant non-polypeptide moiety. For instance, for polymer conjugation to PEG, a frequently used attachment group is the ε-amino group of lysine or the N-terminal amino group. Other polymer attachment groups include a free carboxylic acid group (e.g., that of the C-terminal amino acid residue or of an aspartic acid or glutamic acid residue), suitably activated carbonyl groups, oxidized carbohydrate moieties and mercapto groups. Useful attachment groups and their matching non-peptide moieties are apparent from the table below.

Attachment group	Amino acid	Examples of non-peptide moiety	Conjugation method/- Activated PEG	Reference
—NH$_2$	N-terminal, Lys, His, Arg	Polymer, e.g., PEG, with amide or imine group	mPEG-SPA Tresylated mPEG	Shearwater Inc. Delgado et al., critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249–304 (1992)
—COOH	C-term, Asp, Glu	Polymer, e.g., PEG, with ester or amide group	mPEG-Hz	Shearwater Inc.
		Oligosaccharide moiety	In vitro coupling	
—SH	Cys	Polymer, e.g., PEG, with disulfide, maleimide or vinyl sulfone group	PEG-vinylsulphone PEG-maleimide	Shearwater Inc. Delgado et al., critical reviews in Therapeutic Drug Carrier Systems 9(3,4):249–304 (1992)
		Oligosaccharide moiety	In vitro coupling	
—OH	Ser, Thr, —OH, Lys	Oligosaccharide moiety PEG with ester, ether, carbamate, carbonate	In vivo O-linked glycosylation	
—CONH$_2$	Asn as part of an N-glycosylation site	Oligosaccharide moiety Polymer, e.g., PEG	In vivo N-glycosylation	
Aromatic residue	Phe, Tyr, Trp	Oligosaccharide moiety	In vitro coupling	
—CONH$_2$	Gln	Oligosaccharide moiety	In vitro coupling	Yan and Wold, Biochemistry, 1984, Jul 31; 23(16): 3759–65
Aldehyde Ketone	Oxidized oligo-saccharide	Polymer, e.g., PEG, PEG-hydrazide	PEGylation	Andresz et al., 1978, Makromol. Chem. 179:301, WO 92/16555, WO 00/23114
Guanidino	Arg	Oligosaccharide moiety	In vitro coupling	Lundblad and Noyes, Chemical Reagents for Protein Modification, CRC Press Inc., Florida, USA
Imidazole ring	His	Oligosaccharide moiety	In vitro coupling	As for guanidine

[0034] For in vivo N-glycosylation, the term "attachment group" is used in an unconventional way to indicate the amino acid residues constituting an N-glycosylation site (with the sequence N-X'-S/T/C-X", wherein X' is any amino acid residue except proline, X" any amino acid residue which optionally can be identical to X' and which preferably is different from proline, N is asparagine, and S/T/C is either serine, threonine or cysteine, preferably serine or threonine, and most preferably threonine). Although the asparagine residue of the N-glycosylation site is where the oligosaccharide moiety is attached during glycosylation, such attachment cannot be achieved unless the other amino acid residues of the N-glycosylation site are present. Accordingly, when the non-peptide moiety is an oligosaccharide moiety and the conjugation is to be achieved by N-glycosylation, the term "amino acid residue comprising an attachment group for the non-peptide moiety" as used in connection with alterations of the amino acid sequence of the polypeptide of interest is to be understood as meaning that one or more amino acid residues constituting an N-glycosylation site are to be altered in such a manner that either a functional N-glycosylation site is introduced into the amino acid sequence or removed from said sequence.

[0035] In the present application, amino acid names and atom names (e.g., CA, CB, NZ, N, O, C, etc.) are used as defined by the Protein DataBank (PDB) (www.pdb.org), which is based on the IUPAC nomenclature (IUPAC Nomenclature and Symbolism for Amino Acids and Peptides (residue names, atom names etc.), *Eur. J. Biochem.*, 138, 9-37 (1984) together with their corrections in *Eur. J. Biochem.*, 152, 1 (1985). The term "amino acid residue" is primarily intended to indicate an amino acid residue contained in the group consisting of the 20 naturally occurring amino acids, i.e., alanine (Ala or A), cysteine (Cys or C), aspartic acid (Asp or D), glutamic acid (Glu or E), phenylalanine (Phe or F), glycine (Gly or G), histidine (His or H), isoleucine (Ile or I), lysine (Lys or K), leucine (Leu or L), methionine (Met or M), asparagine (Asn or N), proline (Pro or P), glutamine (Gln or Q), arginine (Arg or R), serine (Ser or S), threonine (Thr or T), valine (Val or V), tryptophan (Trp or W), and tyrosine (Tyr or Y) residues.

[0036] The terminology used for identifying amino acid positions/substitutions is illustrated as follows: E9(a) indicates position number 9 occupied by a glutamic acid residue in the amino acid sequence shown in SEQ ID NO:2. E9(a)N indicates that said glutamic acid residue has been substituted by an asparagine residue. Unless otherwise indicated, the numbering of amino acid residues made herein is made relative to the amino acid sequence shown in SEQ ID NO:2 (for FSH-α, indicated by "(a)") or SEQ ID NO:4 (for FSH-β, indicated by "(b)"). Multiple substitutions are indicated with a "+," e.g., M109(b)N+E111(b)S/T means an amino acid sequence which comprises substitution of the methionine residue in position 109 of FSH-β by an asparagine residue and substitution of the glutamic acid residue in position 111 in FSH-β by a serine or a threonine residue.

[0037] The term "nucleotide sequence" is intended to indicate a consecutive stretch of two or more nucleotide molecules. The nucleotide sequence can be of genomic, cDNA, RNA, semisynthetic, synthetic origin, or any combination thereof.

[0038] The term "polymerase chain reaction" or "PCR" generally refers to a method for amplification of a desired nucleotide sequence in vitro, as described, for example, in U.S. Pat. No. 4,683,195. In general, the PCR method involves repeated cycles of primer extension synthesis, using oligonucleotide primers capable of hybridising preferentially to a template nucleic acid.

[0039] "Cell,""host cell,""cell line" and "cell culture" are used interchangeably herein and all such terms should be understood to include progeny resulting from growth or culturing of a cell. "Transformation" and "transfection" are used interchangeably to refer to the process of introducing DNA into a cell.

[0040] "Operably linked" refers to the covalent joining of two or more nucleotide sequences, by means of enzymatic ligation or otherwise, in a configuration relative to one another such that the normal function of the sequences can be performed. For example, the nucleotide sequence encoding a presequence or secretory leader is operably linked to a nucleotide sequence for a polypeptide if it is expressed as a preprotein that participates in the secretion of the polypeptide; a promoter or enhancer is operably linked to a coding sequence if it affects the transcription of the sequence; a ribosome binding site is operably linked to a coding sequence if it is positioned so as to facilitate translation. Generally, "operably linked" means that the nucleotide sequences being linked are contiguous and, in the case of a secretory leader, contiguous and in reading phase. Linking is accomplished by ligation at convenient restriction sites. If such sites do not exist, then synthetic oligonucleotide adaptors or linkers are used, in conjunction with standard recombinant DNA methods.

[0041] The term "introduce" refers to introduction of an amino acid residue comprising an attachment group for a non-polypeptide moiety, either by substitution of an existing amino acid residue or by insertion of an additional amino acid residue. The term "remove" refers to removal of an amino acid residue comprising an attachment group for a non-polypeptide moiety, either by substitution of the amino acid residue to be removed by another amino acid residue or by deletion (without substitution) of the amino acid residue to be removed.

[0042] When substitutions are performed in relation to a parent polypeptide, they are preferably "conservative substitutions," in other words substitutions performed within groups of amino acids with similar characteristics, e.g., small amino acids, acidic amino acids, polar amino acids, basic amino acids, hydrophobic amino acids and aromatic amino acids.

[0043] Preferred substitutions in the present invention can in particular be chosen from among the conservative substitution groups listed in the table below.

[0044] Conservative Substitution Groups

1	Alanine (A)	Glycine (G)	Serine (S)	Threonine (T)
2	Aspartic acid (D)	Glutamic acid (E)		
3	Asparagine (N)	Glutamine (Q)		
4	Arginine (R)	Histidine (H)	Lysine (K)	
5	Isoleucine (I)	Leucine (L)	Methionine (M)	Valine (V)
6	Phenylalanine (F)	Tyrosine (Y)	Tryptophan (W)	

[0045] The term "immunogenicity" as used in connection with a given substance is intended to indicate the ability of the substance to induce a response from the immune system. The immune response can be a cell or antibody mediated response (see, e.g., Roitt: Essential Immunology (8th Edition, Blackwell) for further definition of immunogenicity). Normally reduced antibody reactivity will be an indication of a reduced immunogenicity. The reduced immunogenicity can be determined by use of any suitable method known in the art, e.g., in vivo or in vitro.

[0046] The term "functional in vivo half-life" is used in its normal meaning, i.e., the time at which 50% of the biological activity of the polypeptide or conjugate is still present in the body/target organ, or the time at which the activity of the polypeptide or conjugate is 50% of the initial value. As an alternative to determining functional in vivo half-life, "serum half-life" can be determined, i.e., the time at which 50% of the dispensed polypeptide or conjugate molecules is still present in the circulation/plasma/bloodstream. The magnitude of serum half-life is usually a good indication of the magnitude of functional in vivo half-life. Alternative terms to serum half-life include "plasma half-life,""circulating half-life,""serum clearance,""plasma clearance" and "clearance half-life." The polypeptide or conjugate is cleared by the action of one or more of the kidney, reticuloendothelial systems (RES), spleen or liver, by FSH-receptor-mediated elimination, or by specific or non-specific proteolysis. Normally, clearance depends on size (relative to the cutoff for glomerular filtration), charge, attached carbohydrate chains, and the presence of cellular receptors for the protein. The functional in vivo half-life and the serum half-life can be determined by any suitable method known in the art as further discussed in the Examples section hereinafter.

[0047] The term "increased" as used about the functional in vivo half-life or serum half-life is used to indicate that the relevant half-life of the conjugate or polypeptide is statistically significantly increased relative to that of a reference molecule, such as a non-conjugated rhFSH (recombinant human FSH), e.g., Gonal-F® (available from Serono) or Puregon® (available from Organon), as determined under comparable conditions. For instance, the relevant half-life can be increased by at least about 25%, such as by at least about 50%, e.g., by at least about 100%, 200% or 500%.

[0048] The term "renal clearance" is used in its normal meaning to indicate any clearance taking place by the kidneys, e.g., by glomerular filtration, tubular excretion or tubular elimination. Renal clearance depends on physical characteristics of the conjugate, including size (diameter), symmetry, shape/rigidity and charge. Reduced renal clearance can be established by any suitable assay, e.g., an established in vivo assay. Typically, renal clearance is determined by administering a labelled (e.g., radioactive or fluorescent labelled) polypeptide conjugate to a patient and measuring the label activity in urine collected from the patient. Reduced renal clearance is determined relative to a corresponding reference polypeptide, e.g., the corresponding non-conjugated polypeptide, a non-conjugated corresponding wild-type polypeptide or another conjugated polypeptide (such as a conjugated polypeptide not according to the invention), under comparable conditions.

[0049] In some cases, it will be preferred to obtain a clearance that is only slightly reduced (i.e., total clearance by renal clearance, receptor-mediated clearance and/or other clearance mechanisms), e.g., to increase the in vivo half-life from about 24 hours to about 3-4 days, while in other cases a longer half-life of e.g., about 6-7 days will be desired. As will be explained in further detail below, the number and size of such polymer molecules can be adapted in order to obtain a desired clearance, as well as other desired properties, suitable for a given clinical indication. Preferably, the conjugate of the invention has a reduced clearance of at least about 50%, such as least about 75% or at least about 90%, as compared to the corresponding non-conjugated polypeptide (such as hFSH or rhFSH) as determined under comparable conditions.

[0050] Generally, activation of the receptor is coupled to receptor-mediated clearance (RMC) such that binding of a polypeptide to its receptor without activation does not lead to RMC, while activation of the receptor leads to RMC. The clearance is due to internalisation of the receptor-bound polypeptide with subsequent lysosomal degradation. Reduced RMC can therefore be achieved by designing the conjugate so as to be able to bind and activate a sufficient number of receptors to obtain optimal in vivo biological response and avoid activation of more receptors than required for obtaining such response, e.g., by substitution, polymer conjugation or other modification of one or more amino acid residues located at or near a receptor-binding site. This can be reflected in reduced in vitro bioactivity and/or increased off-rate.

[0051] The term "FSH-α" is intended to indicate a polypeptide having qualitatively similar functions or activities as the corresponding wildtype FSH α subunit, including the capability of forming a dimeric polypeptide with an FSH-β subunit (FSH-β), which dimeric polypeptide exhibits FSH activity. Alternatively used terms include "FSH-α polypeptide,""FSH-α subunit," and "modified FSH-α." Analogously, the term "FSH-β" is intended to indicate a polypeptide having qualitatively similar functions or activities as the corresponding wildtype FSH β subunit, including the capability of dimerizing with FSH-α and thereby forming a dimeric polypeptide exhibiting FSH activity. Alternatively used terms include "FSH-β polypeptide,""FSH-β subunit," and "modified FSH-β."

[0052] The term "exhibiting FSH activity" is intended to indicate that the conjugate or polypeptide has one or more of the functions of wildtype FSH, in particular hFSH, including the capability of binding to and activating an FSH receptor. The FSH activity is conveniently assayed using the in vitro activity assay described in the Examples section below. The conjugate or polypeptide "exhibiting" FSH activity is considered to have such activity when it displays a measurable function, e.g., a measurable activity. The dimeric polypeptide exhibiting FSH activity can also be termed "FSH molecule" herein.

[0053] Conjugate of the Invention

[0054] As stated above, in a first aspect, the invention relates to a polypeptide conjugate exhibiting FSH activity, comprising i) a polypeptide comprising FSH-α and FSH-β subunits, wherein at least one of the FSH-α and FSH-β subunits differs from the corresponding wildtype subunit in at least one introduced or removed amino acid residue

comprising an attachment group for non-polypeptide moiety, and ii) a non-polypeptide moiety bound to an attachment group of the polypeptide. Examples of amino acid residues that can be introduced and/or removed are described in further detail in the following sections.

[0055] By removing and/or introducing an amino acid residue comprising an attachment group for the non-polypeptide moiety, it is possible to specifically adapt the polypeptide so as to make the molecule more susceptible to conjugation to the non-polypeptide moiety of choice, to optimize the conjugation pattern (e.g., to ensure an optimal distribution of non-polypeptide moieties on the surface of the FSH molecule and to ensure that only the attachment groups intended to be conjugated are present in the molecule) and thereby obtain a new conjugate molecule which has FSH activity and in addition one or more improved properties as compared to FSH molecules available today, in particular increased functional in vivo half-life and/or reduced clearance.

[0056] In the conjugate of the invention, one or both of the FSH subunits can be modified according to the invention. For instance, the amino acid sequence of FSH-α can be modified as described herein, whereas FSH-β is unmodified, and vice versa. Alternatively, both of FSH-α and FSH-β can be modified according to the invention.

[0057] While the FSH-α and/or FSH-β can be of any origin, it is in particular of mammalian origin, and preferably of human origin. Accordingly, the corresponding wild-type subunits referred to above are preferably hFSH-α and hFSH-β, respectively, with the amino acid sequences shown in SEQ ID NO:2 and 4.

[0058] In a preferred embodiment, one difference between the amino acid sequence of FSH-α and/or FSH-β and the corresponding wildtype sequence is that at least one and preferably more, e.g., 1-20, amino acid residues comprising an attachment group for the non-polypeptide moiety have been introduced, by insertion or substitution, into the amino acid sequence. Thereby, properties such as the molecular weight, shape, size and/or charge of the conjugate can be optimised. Preferably, such amino acid residues are introduced in positions occupied by an amino acid residue having more than 25%, more preferably more than 50%, such as more than 75% of its side chain exposed at the surface of the molecule.

[0059] The term "one difference" as used in the present application is intended to allow for additional differences being present. Accordingly, in addition to the specified amino acid difference, other amino acid residues than those specified can be mutated.

[0060] In one embodiment, one difference between the amino acid sequence of FSH-α and/or FSH-β and that of the corresponding wildtype polypeptide is that at least one and possible more, e.g., 1-15, amino acid residues comprising an attachment group for the non-polypeptide moiety have been removed, by substitution or deletion, from the amino acid sequence. The amino acid residue to be removed is preferably one to which conjugation is disadvantageous, e.g., an amino acid residue located at or near a functional site of the polypeptide (since conjugation at such a site can result in inactivation or reduced FSH activity of the resulting conjugate due to impaired receptor recognition). In the present

context the term "functional site" is intended to indicate one or more amino acid residues which are essential for or otherwise involved in the function or performance of hFSH, in particular dimerization and/or receptor binding and activation. Such amino acid residues are a part of a functional site. The functional site can be determined by methods known in the art and is preferably identified by analysis of a structure of the polypeptide complexed to a relevant receptor, such as the hFSH receptor.

[0061] In another embodiment, the alteration of FSH-α and/or FSH-β embraces removal as well as introduction of amino acid residues comprising an attachment group for the non-polypeptide moiety of choice.

[0062] In order to avoid too much disruption of the structure and function of the FSH molecule, the total number of amino acid residues to be altered in accordance with the present invention will typically not exceed 20 for each individual subunit. Preferably, the polypeptide part of the conjugate of the invention or the dimeric polypeptide of the invention comprises an amino acid sequence which differs in a total of 1-20 amino acid residues from the amino acid sequences shown in SEQ ID NO:2 and/or SEQ ID NO:4, such as in 1-15 or 2-12 amino acid residues, e.g., in 3-10 amino acid residues. Thus, normally the polypeptide part of the conjugate or the dimeric polypeptide of the invention comprises an amino acid sequence which in total differs from the amino acid sequences shown in SEQ ID NO:2 and/or SEQ ID NO:4 in 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19 or 20 amino acid residues.

[0063] The FSH-α and/or FSH-β subunits of the dimeric polypeptide are preferably any of the specific modified FSH-α and/or FSH-β polypeptides disclosed in the subsequent sections having introduced and/or removed amino acid residues comprising an attachment group for the relevant non-polypeptide moiety.

[0064] The amino acid residue comprising an attachment group for a non-polypeptide moiety, whether it is removed or introduced, is selected on the basis of the nature of the non-polypeptide moiety of choice and, in most instances, on the basis of the method in which conjugation between the polypeptide and the non-polypeptide moiety is to be achieved. It will be understood that in order to preserve a measurable function of the modified FSH-α and/or FSH-β, amino acid residues to be modified (by deletion or substitution) are selected from those amino acid residues which are not essential for providing a measurable activity. Accordingly, amino acid residues to be modified are different from those required for subunit dimerization and/or receptor binding or activation. The identity of such amino acid residues is described in the art (e.g., references identified in the Background section above) or can be determined by a person skilled in the art using methods known in the art.

[0065] In addition to the amino acid alterations disclosed herein aimed at introducing and/or removing attachment sites for the non-polypeptide moiety, the FSH-α and/or FSH-β subunits can comprise other amino acid alterations that need not be related to introduction or removal of attachment sites, i.e., other substitutions, insertions or deletions. These may, for example, include truncation of the N- and/or C-terminus by one or more amino acid residues, or addition of one or more extra residues at the N- and/or C-terminus. Examples of such additional amino acid

changes include adding part of or the entire CTP region of hCG to the C-terminus of FSH-α or introducing any other mutation (in particular selected among those reported to enhance FSH activity and/or increase the functional in vivo half-life, cf. the Background of the Invention section herein). In such cases, the amino acid sequence of the basic polypeptide subunits, i.e., the sequence of the subunits excluding any introduced or removed attachment sites, will typically have a degree of homology, compared to the relevant wildtype sequence (normally hFSH-α or hFSH-β), of at least about 80%, more typically at least about 90%, such as at least about 95%. Amino acid sequence homology/identity is conveniently determined from aligned sequences, using e.g., the ClustalW program or from the PFAM families database version 4.0 (http://pfam.wustl.edu/) (*Nucleic Acids Res. Jan.* 1, 1999; 27(1):260-2) by use of GENEDOC version 2.5 (Nicholas, K. B., Nicholas H. B. Jr., and Deerfield, D. W. II. 1997 GeneDoc: Analysis and Visualization of Genetic Variation, EMBNEW.NEWS 4:14; Nicholas, K. B. and Nicholas H. B. Jr. 1997 GeneDoc: Analysis and Visualization of Genetic Variation).

[0066] Preferably, the conjugate of the present invention has one or more improved properties as compared to hFSH, including increased functional in vivo half-life, increased serum half-life, reduced renal clearance, reduced receptor-mediated clearance, reduced immunogenicity and/or an increased bioavailability as compared to rhFSH (e.g., Gonal-F® or Puregon®). Consequently, medical treatment with a conjugate of the invention offers advantages over the currently available FSH compounds, in particular longer duration between injections.

[0067] Conjugate of the Invention wherein the Non-polypeptide Moiety is an Oligosaccharide Moiety

[0068] It has been found that N-glycosylation is important for FSH activity and also that the extent and type of oligosaccharide moiety attached by in vivo glycosylation is important for functional in vivo half-life of the glycosylated FSH. In order to obtain a different, increased glycosylation it is desirable to introduce at least one glycosylation site. Accordingly, in a preferred aspect, the invention relates to a heterodimeric polypeptide conjugate exhibiting FSH activity comprising a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein the amino acid sequence of at least one of the FSH-α and FSH-β subunits differs from that of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced, and having at least one oligosaccharide moiety bound to an N-glycosylation site of at least one of the subunits.

[0069] A suitable N-glycosylation site can be introduced by introducing, by substitution or insertion, an asparagine residue in a position occupied by an amino acid residue having more than 25% of its side chain exposed at the surface of the polypeptide, which position does not have a proline residue located in position +1 or +3 therefrom. If the amino acid residue located in position +2 is a serine or threonine, no further amino acid substitution is required. However, if this position is occupied by a different amino acid residue, a serine or threonine residue needs to be introduced.

[0070] A preferred conjugate, according to this embodiment, is one which comprises a modified FSH-α subunit having an amino acid residue which differs from that of hFSH-α in the introduction of at least one N-glycosylation site by means of a mutation selected from the group con-

sisting of P2(a)N+V4(a)S, P2(a)N+V4(a)T, D3(a)N+Q5(a)S, D3(a)N+Q5(a)T, V4(a)N+D6(a)S, V4(a)N+D6(a)S, D6(a)N+P8(a)S, D6(a)N+P8(a)T, E9(a)N+T11(a)S, E9(a)N, T11(a)N+Q13(a)S, T11(a)N+Q13(a)T, L12(a)N+E14(a)S, L12(a)N+E14(a)T, E14(a)N+P16(a)S, E14(a)N+P16(a)T, P16(a)N+F18(a)S, P16(a)N+F18(a)T, F17(a)N, F17(a)N+S19(a)T, G22(a)N+P24(a)S, G22(a)N+P24(a)T, P24(a)N+L26(a)S, P24(a)N+L26(a)T, F33(a)N+R35(a)S, F33(a)N+R35(a)T, R42(a)N+K44(a)S, R42(a)N+K44(a)T, S43(a)N+K45(a)S, S43(a)N+K45(a)T, K44(a)N+T46(a)S, K44(a)N, K45(a)N+M47(a)S, K45(a)N+M47(a)T, T46(a)N+L48(a)S, T46(a)N+L48(a)T, L48(a)N+Q50(a)S, 148(a)N+Q50(a)T, V49(a)N+K51(a)S, V49(a)N+K51(a)T, Q50(a)N+N52(a)S, Q50(a)N+N52(a)T, V61(a)N+K63(a)S, V61(a)N+K63(a)T, K63(a)N+Y65(a)S, K63(a)N+Y65(a)T, S64(a)N+N66(a)S, S64(a)N+N66(a)T, Y65(a)N+R67(a)S, Y65(a)N+R67(a)T, V68(a)S, V68(a)T, R67(a)N+T69(a)S, R67(a)N, T69(a)N+M71(a)S, T69(a)N+M71(a)T, M71(a)N+G73(a)S, M71(a)N+G73(a)T, G72(a)N+F74(a)S, G72(a)N+F74(a)T, G73(a)N+K75(a)S, G73(a)N+K75(a)T, F74(a)N+V76(a)S, F74(a)N+V76(a)T, K75(a)N+E77(a)S, K75(a)N+E77(a)T, A81(a)N+H83(a)S, A81(a)N+H83(a)T, H83(a)N, T86(a)N+Y88(a)S, T86(a)N+Y88(a)T, Y88(a)N+H90(a)S, Y88(a)N+H90(a)T, Y89(a)N+K91(a)S, Y89(a)N+K91(a)T, H90(a)N and H90(a)N+S92(a)T (positions with more than 25% side chain exposure). Among these possible positions for mutation, more preferred mutations are those where a glycosylation site can be introduced by mutation of a single amino acid residue, i.e., selected from the group consisting of V68(a)S, V68(a)T, E9(a)N, F17(a)N, K44(a)N, R67(a)N, H83(a)N and H90(a)N.

[0071] More preferably, a glycosylation site is introduced at a position having more than 50% side chain exposure, i.e., by means of a mutation selected from the group consisting of P2(a)N+V4(a)S, P2(a)N+V4(a)T, D3(a)N+Q5(a)S, D3(a)N+Q5(a)T, V4(a)N+D6(a)S, V4(a)N+D6(a)S, D6(a)N+P8(a)S, D6(a)N+P8(a)T, E9(a)N+T11(a)S, E9(a)N, T11(a)N+Q13(a)S, T11(a)N+Q13(a)T, E14(a)N+P16(a)S, E14(a)N+P16(a)T, P16(a)N+F18(a)S, P16(a)N+F18(a)T, F17(a)N, F17(a)N+S19(a)T, G22(a)N+P24(a)S, G22(a)N+P24(a)T, K45(a)N+M47(a)S, K45(a)N+M47(a)T, T46(a)N+L48(a)S, T46(a)N+L48(a)T, L48(a)N+Q50(a)S, 148(a)N+Q50(a)T, V49(a)N+K51(a)S, V49(a)N+K51(a)T, Q50(a)N+N52(a)S, Q50(a)N+N52(a)T, K63(a)N+Y65(a)S, K63(a)N+Y65(a)T, S64(a)N+N66(a)S, S64(a)N+N66(a)T, V68(a)S, V68(a)T, R67(a)N+T69(a)S, R67(a)N, T69(a)N+M71(a)S, T69(a)N+M71(a)T, G72(a)N+F74(a)S, G72(a)N+F74(a)T, G73(a)N+K75(a)S, G73(a)N+K75(a)T, K75(a)N+E77(a)S, K75(a)N+E77(a)T, T86(a)N+Y88(a)S, T86(a)N+Y88(a)T, Y89(a)N+K91(a)S, Y89(a)N+K91(a)T, H90(a)N, and H90(a)N+S92(a)T. Still more preferably, glycosylation sites are introduced via mutation of a single amino acid residue selected from the group consisting of E9(a)N, F17(a)N, R67(a)N, and H90(a)N.

[0072] The FSH-β part of such conjugates with an altered FSH-α subunit can be hFSH-β or any of the modified FSH-β polypeptides described herein.

[0073] Alternatively or additionally, the conjugate according to this embodiment comprises a modified FSH-β having an amino acid residue which differs from that of hFSH-β in the introduction of at least one N-glycosylation site by a mutation selected from the group consisting of S2(b)N+E4(b)S, S2(b)N+E4(b)T, E4(b)N+T6(b)S, E4(b)N, L5(b)N+N7(b)S, L5(b)N+L7(b)T, T6(b)N+I8(b)S, T6(b)N+I8(b)T, I8(b)N+I10(b)S, I8(b)S, I8(b)N+I10(b)T, T9(b)N+A11(b)S,

T9(b)N+A11(b)T, K14(b)N+E16(b)S, K14(b)N+E16(b)T, F19(b)N+I21(b)S, F19(b)N+I21(b)T, I21(b)N+I23(b)S, I21(b)N+I23(b)T, S22(b)N+N24(b)S, S22(b)N+N24(b)T, Y31(b)N+Y33(b)S, Y31(b)N+Y33(b)T, Y33(b)N+R35(b)S, Y33(b)N+R35(b)T, R35(b)N+L37(b)S, R35(b)N+L37(b)T, D36(b)N+V38(b)S, D36(b)N+V38(b)T, L37(b)N+Y39(b)S, L37(b)N+Y39(b)T, K40(b)N+P42(b)S, K40(b)N+P42(b)T, A43(b)N+P45(b)S, A43(b)N+P45(b)T, P45(b)N+I47(b)S, P45(b)N+I47(b)T, K46(b)N+Q48(b)S, K46(b)N+Q48(b)T, I47(b)N+K49(b)S, I47(b)N+K49(b)T, K54(b)N+L56(b)S, K54(b)N+L56(b)T, E55(b)N+V57(b)S, E55(b)N+V57(b)T, L56(b)N+Y58(b)S, L56(b)N+Y58(b)T, V57(b)N+E59(b)S, V57(b)N+E59(b)T, Y58(b)N+T60(b)S, Y58(b)N, E59(b)N+ V61(b)S, E59(b)N+V61(b)T, T60(b)N+R62(b)S, T60(b)N+ R62(b)T, R62(b)N+P64(b)S, R62(b)N+P64(b)T, G65(b)N+ A67(b)S, G65(b)N+A67(b)T, A67(b)N+H69(b)S, A67(b)N+H69(b)T, H68(b)N+A70(b)S, H68(b)N+A70(b)T, H69(b)N+D71(b)S, H69(b)N+D71(b)T, D71(b)N+L73(b)S, D71(b)N+L73(b)T, L73(b)N+T75(b)S, L73(b)N, T75(b)N+ P77(b)S, T75(b)N+P77(b)T, H83(b)N+G85(b)S, H83(b)N+ G85(b)T, K86(b)N+D88(b)S, K86(b)N+D88(b)T, D88(b)N+D90(b)S, D88(b)N+D90(b)T, S89(b)N, S89(b)N+ S91(b)T, D90(b)N+T92(b)S, D90(b)N, S91(b)N+D93(b)S, S91(b)N+D93(b)T, D93(b)N+T96(b)S, D93(b)N, T95(b)N+ R97(b)S, T95(b)N+R97(b)T, V96(b)N+G98(b)S, V96(b)N+ G98(b)T, R97(b)N+L99(b)S, R97(b)N+L99(b)T, L99(b)N+ P101(b)S, L99(b)N+P101(b)T, Y103(b)N, Y103(b)N+ S105(b)T, S105(b)N+G107(b)S, S105(b)N+G107(b)T, F106(b)N+E108(b)S, F106(b)N+E108(b)T, G107(b)N+ M109(b)S, G107(b)N+M109(b)T, E108(b)N+K110(b)S, E108(b)N+K110(b)T, M109(b)N+E111(b)S, and M109(b)N+E111(b)T (mutations at positions with at least 25% side chain exposure). Preferably, glycosylation sites are introduced by means of mutation of a single amino acid residue selected from the group consisting of E4(b)N, Y58(b)N, L73(b)N, S89(b)N, D90(b)N, D93(b)N, and Y103(b)N.

[0074] More preferably, a modified FSH-β has an amino acid residue which differs from that of hFSH-β in the introduction of at least one N-glycosylation site by a mutation selected from the group consisting of F19(b)N+I21(b)S, F19(b)N+I21(b)T, Y33(b)N+R35(b)S, Y33(b)N+R35(b)T, A43(b)N+P45(b)S, A43(b)N+P45(b)T, P45(b)N+I47(b)S, P45(b)N+I47(b)T, K46(b)N+Q48(b)S, K46(b)N+Q48(b)T, I47(b)N+K49(b)S, I47(b)N+K49(b)T, K54(b)N+L56(b)S, K54(b)N+L56(b)T, E55(b)N+V57(b)S, E55(b)N+V57(b)T, V57(b)N+E59(b)S, V57(b)N+E59(b)T, Y58(b)N+T60(b)S, Y58(b)N, E59(b)N+V61(b)S, E59(b)N+V61(b)T, R62(b)N+P64(b)S, R62(b)N+P64(b)T, G65(b)N+A67(b)S, G65(b)N+A67(b)T, A67(b)N+H69(b)S, A67(b)N+H69(b)T, H68(b)N+A70(b)S, H68(b)N+A70(b)T, H69(b)N+D71(b)S, H69(b)N+D71(b)T, D71(b)N+L73(b)S, D71(b)N+L73(b)T, L73(b)N+T75(b)S, L73(b)N, T75(b)N+P77(b)S, T75(b)N+ P77(b)T, H83(b)N+G85(b)S, H83(b)N+G85(b)T, K86(b)N+ D88(b)S, K86(b)N+D88(b)T, D88(b)N+D90(b)S, D88(b)N+D90(b)T, S89(b)N, S89(b)N+S91(b)T, D90(b)N+ T92(b)S, D90(b)N, S91(b)N+D93(b)S, S91(b)N+D93(b)T, T95(b)N+R97(b)S, T95(b)N+R97(b)T, R97(b)N+L99(b)S, R97(b)N+L99(b)T, L99(b)N+P101(b)S, L99(b)N+ P101(b)T, Y103(b)N, Y103(b)N+S105(b)T, S105(b)N+ G107(b)S, S105(b)N+G107(b)T, F106(b)N+E108(b)S, F106(b)N+E108(b)T, G107(b)N+M109(b)S, G107(b)N+ M109(b)T, E108(b)N+K110(b)S, E108(b)N+K110(b)T, M109(b)N+E111(b)S, and M109(b)N+E111(b)T (positions

having more than 50% side chain accessibility). Among these positions, it is preferred to introduce glycosylation sites using mutation of a single amino acid residue selected from the group consisting of Y58(b)N, L73(b)N, S89(b)N, D90(b)N, and Y103(b)N.

[0075] The FSH-α part of such conjugates with an altered FSH-β subunit can be hFSH-α or any of the modified FSH-α polypeptides described herein.

[0076] The FSH-α and/or FSH-β polypeptide can further differ from hFSH-α and/or hFSH-β in at least one removed, naturally occurring N-glycosylation site. In particular, FSH-α can comprise a substitution of N78(a) and/or T80(a) by any other amino acid residue and/or FSH-β can comprise a substitution of N7(b), T9(b), N24(b) and/or T26(b) by any other amino acid residue. Preferably, the N residue is substituted by Q or D, and the T residue by A or G.

[0077] Furthermore, one or both of the FSH-α and FSH-β subunits of the conjugate according to this embodiment (having at least one of the above mentioned N-glycosylation site modifications) can differ from hFSH-α and hFSH-β, respectively, in the removal, preferably by substitution, of at least one lysine residue. See the section below on removal of lysine residues for further details.

[0078] An alternative embodiment of this aspect of the invention is one in which at least one of said FSH-α and FSH-β subunits comprises at least one introduced N- or O-glycosylation site at the N-terminal thereof, and wherein the at least one introduced glycosylation site is glycosylated; see the discussion of peptide addition below. In this case, the respective subunits can comprise one or more of the modifications disclosed elsewhere herein, or one or both of the subunits can be the respective wildtype subunits, but having the at least one introduced terminal glycosylation site. Thus, the polypeptide conjugate can be one in which the FSH-α subunit comprises hFSH-α having the sequence shown in SEQ ID NO:2, and/or in which the FSH-β subunit comprises hFSH-β having the sequence shown in SEQ ID NO:4. In a particular embodiment, both of the subunits correspond to the respective wildtype hFSH subunits, although with either the α or β subunit, or both, having an introduced N-terminal glycosylation site.

[0079] The introduced glycosylation site can be of the type described elsewhere herein; see the discussion of glycosylation under the general discussion of attachment groups above. A non-limiting example of a suitable glycosylation site for introduction at the N-terminal is the sequence Ala-Asn-Ile-Thr-Val-Asn-Ile-Thr-Val, e.g., for insertion of two glycosylation sites upstream of a mature FSH-α or FSH-β sequence.

[0080] Introduction of glycosylation sites by means of peptide addition

[0081] In addition to or as an alternative to introducing glycosylation sites within the amino acid sequence of one or both of the subunits, one or more additional glycosylation sites can be introduced by means of a "peptide addition" as discussed in the following. In this case, each of the polypeptide subunits comprises or consists of or consists essentially of the primary structure,

$$\mathrm{NH_2-X-P-COOH \ or \ NH_2-P-X-COOH,}$$
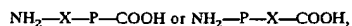
[0082] wherein

[0083] X is a peptide addition comprising or contributing to a glycosylation site, and P is the basic polypeptide subunit to be modified, i.e., FSH-α or FSH-β, e.g., a wildtype polypeptide subunit as defined herein or a modified polypeptide having introduced and/or removed glycosylation sites or other attachment sites in the mature part of the polypeptide.

[0084] In the context of a peptide addition the term "comprising a glycosylation site" is intended to mean that a complete glycosylation site is present in the peptide addition, whereas the term "contributing to a glycosylation site" is intended to cover the situation where at least one amino acid residue of an N-glycosylation site is present in the peptide addition while the other amino acid residue of said site is present in the polypeptide P, whereby the glycosylation site can be considered to bridge the peptide addition and the polypeptide.

[0085] Usually, the peptide addition is fused to the N-terminal or C-terminal end of the polypeptide P as reflected in the above shown structure so as to provide an N- or C-terminal elongation of the polypeptide P, preferably at the N-terminal. However, it is also possible to insert the peptide addition within the amino acid sequence of the polypeptide P whereby the polypeptide comprises, consists of or consists essentially of the primary structure NH_2—P_x—X—P_y—COOH, wherein

[0086] P_x is an N-terminal part of the relevant polypeptide P,

[0087] P_y is a C-terminal part of said polypeptide P, and

[0088] X is a peptide addition comprising or contributing to a glycosylation site.

[0089] In order to minimize structural changes effected by the insertion of the peptide addition within the sequence of the polypeptide P, it is desirable that it be inserted in a non-structural part thereof. For instance, P_x can be a non-structural N-terminal part of a mature polypeptide P, and P_y a structural C-terminal part of said mature polypeptide, or P_x can be a structural N-terminal part of a mature polypeptide P, and P_y a non-structural C-terminal part of said mature polypeptide.

[0090] The term "non-structural part" is intended to indicate a part of either the C- or N-terminal end of the folded polypeptide subunit that is outside the first structural element, such as an α-helix or a β-sheet structure. The non-structural part can easily be identified in a three-dimensional structure or model of the polypeptide. If no structure or model is available, a non-structural part typically comprises or consists of the first or last 1-20 amino acid residues, such as 1-10 amino acid residues of the amino acid sequence constituting the mature form of the polypeptide.

[0091] When the peptide addition comprises only few amino acid residues, e.g., 1-5, such as 1-3 amino acid residues, and in particular one amino acid residue, the peptide addition can be inserted into a loop structure of the polypeptide P and thereby elongate the loop.

[0092] In principle, the peptide addition X can be any stretch of amino acid residues ranging from a single amino acid residue to a mature protein. In the present context, it is contemplated that each peptide addition will normally comprise up to about 50 amino acid residues, such as 2-30 or

3-20 amino acid residues. The peptide addition can be designed by a site-specific or random approach. In order to minimize the risk of an immunogenic response, however, it is preferable to select N- or C-terminal extensions of the FSH sequence that comprise peptide sequences that are part of naturally occurring human proteins. Non-limiting examples of such peptide sequences include the sequence NSTQNATA, which corresponds to positions 231 to 238 of the human calcium activated channel 2 precursor (to add two N-glycosylation sites to FSH), or the sequence ANLTVRN-LTRNVTV, which corresponds to positions 538 to 551 of the human G protein coupled receptor 64 (to add three N-glycosylation sites to FSH).

[0093] Typically, each peptide addition X comprises 1-10 glycosylation sites. The peptide addition X can thus comprise 1, 2, 3, 4, 5, 6, 7, 8, 9 or 10 glycosylation sites. It is well known that a frequently occurring consequence of modifying an amino acid sequence of, e.g., a human protein is that new epitopes are created by such modification. Non-polypeptide moieties can be used to shield any new epitopes created by the peptide addition, and therefore it is desirable that sufficient glycosylation sites (or attachment groups for another non-polypeptide moiety, e.g., a polymer such as PEG) are present to enable shielding of all epitopes introduced into the sequence. This is e.g., achieved when the peptide addition X comprises at least one glycosylation site within a stretch of 30 contiguous amino acid residues, preferably as at least one glycosylation sites within 20 amino acid residues, more preferably at least one attachment group within 10 amino acid residues, in particular 1-3 attachment groups within a stretch of 10 contiguous amino acid residues in the peptide addition X.

[0094] Preferably, the glycosylation site of the peptide addition is an in vivo glycosylation site, preferably an N-glycosylation site. For instance, the peptide addition X can have the structure X_1-N-X_2-T/S/C-Z, wherein X_1 is a peptide comprising at least one amino acid residue or is absent, X_2 is any amino acid residue different from P, and Z is absent or is a peptide comprising at least one amino acid residue. For instance, X_1 can be absent, X_2 can be an amino acid residue selected from the group consisting of I, A, G, V and S (all relatively small amino acid residues), and Z can comprise at least 1 amino acid residue. Z can e.g., be a peptide comprising up to 50 amino acid residues and e.g., up to 10 glycosylation sites.

[0095] Alternatively, X_1 can comprise at least one amino acid residue, e.g., 1-50 amino acid residues with 1-10 glycosylation sites, X_2 can be an amino acid residue selected from the group consisting of I, A, G, V and S, and Z can be absent.

[0096] Examples of peptide additions for use in the present invention are ANITVNITV, NDTVNFT and NITVNITV; see Examples 9 and 10 below, which illustrate addition of these sequences at the N-terminal of the FSH-α and β subunits.

[0097] The peptide addition can comprise one or more of these peptide sequences, i.e., at least two of said sequences either directly linked together or separated by one or more amino acid residues, or can contain two or more copies of any of these peptide sequence. It will be understood that the above specific sequences are given for illustrative purposes and thus do not constitute an exhaustive list of peptide sequences of use in the present invention.

[0098] In one embodiment, the peptide addition X has an N residue in position −2 or −1, and the polypeptide P or P_x has a T or an S residue in position +1 or +2, respectively, the residue numbering being made relative to the N-terminal amino acid residue of P or P_x, whereby an N-glycosylation site is formed. For instance, the polypeptide can have a T or S residue in position 2, preferably a T residue, and the peptide addition is AN or comprises AN as the C-terminal amino acid residues.

[0099] O-glycosylation

[0100] As an alternative or in addition to the mutations discussed above, the heterodimeric polypeptide can comprise one or more introduced O-glycosylation sites, for example the amino acid sequence AATPAP, which has been found to be an efficient signal sequence for O-glycosylation in vivo (Asada et al. (1999) *Glycoconj. J.* 16(7):321-6). The AATPAP sequence for O-glycosylation is preferably introduced by way of insertion at the N- and/or C-terminus of the FSH-α and/or FSH-β subunit.

[0101] Preparation of glycosylated conjugates

[0102] It will be understood that in order to prepare a conjugate according to this aspect, the polypeptide must be expressed in a glycosylating host cell capable of attaching oligosaccharide moieties at the glycosylation site(s) in vivo or alternatively subjected to in vitro glycosylation. Examples of glycosylating host cells are given in the section further below entitled "Coupling to an oligosaccharide moiety."

[0103] In addition to an oligosaccharide moiety, the conjugate according to the aspect of the invention described in the present section can contain additional non-polypeptide moieties different from O-linked or N-linked oligosaccharide moieties, in particular a polymer molecule such as PEG as described herein conjugated to one or more attachment groups present in the polypeptide part of the conjugate. This is particularly relevant when a lysine residue (or any other amino acid residue comprising an attachment group for the polymer molecule in question) has been introduced and/or removed.

[0104] It will be understood that any of the amino acid changes specified in this section can be combined with any of the amino acid changes specified in the other sections herein disclosing specific amino acid changes.

[0105] Conjugate of the Invention wherein the Non-polypeptide Moiety is Attached to a Lysine or the N-terminal Amino Acid Residue

[0106] In a further preferred embodiment, the conjugate of the invention is one wherein the amino acid residue comprising an attachment group for the non-polypeptide moiety is a lysine residue and the non-polypeptide moiety is any molecule which has lysine as an attachment group. For instance, the non-polypeptide moiety can be a polymer molecule, in particular any of the molecules mentioned in the section entitled "Conjugation to a polymer molecule," and preferably selected from the group consisting of linear or branched polyethylene glycol and polyalkylene oxide. Most preferably, the polymer molecule is mPEG-SPA or oxycarbonyl-oxy-N-dicarboxyimide PEG (U.S. Pat. No. 5,122,614).

[0107] The FSH-α and/or FSH-β having introduced and/or removed at least one lysine can advantageously be in vivo glycosylated, e.g., using naturally occurring glycosylation sites present in the relevant FSH polypeptide. However, in a particular embodiment, the conjugate is one wherein the amino acid sequence of FSH-α and/or FSH β differs from that of FSH-α and/or FSH-β in that an N-glycosylation site has been introduced and/or removed. Such introduced/removed sites can be any of those described in the section entitled "Conjugate of the invention wherein the non-polypeptide moiety is an oligosaccharide moiety."

[0108] i) Removal of lysine residues

[0109] hFSH-α contains 6 lysine residues and hFSH-β 7. In order to avoid conjugation to one or more of these lysine residues, e.g., lysine residues located at or close to the receptor-binding site of hFSH, it can be desirable to remove at least one lysine residue. Accordingly, in one embodiment, the conjugate of the invention is one which comprises a modified FSH-α having an amino acid residue which differs from that of hFSH-α in the removal of at least one lysine residue selected from the group consisting of K44(a), K45(a), K51(a), K63(a), K75(a), and K91(a), in particular at least one amino acid residue selected from of the group consisting of K44(a), K45(a), K63(a), K75(a), and K91(a) (these residues having more than 25% of their side chain exposed to the surface), and preferably from the group consisting of K45(a), K63(a), K75(a), and K91(a) (these residues having more than 50% of their side chain exposed to the surface). The FSH-β part of this conjugate can be hFSH-β or any of the modified FSH-β polypeptides described herein.

[0110] In another embodiment, the conjugate of the invention is one which comprises a modified FSH-β having an amino acid residue which differs from that of hFSH-β in the removal of at least one lysine residue selected from the group consisting of K14(b), K40(b), K46(b), K49(b), K54(b), K86(b), and K110(b), in particular at least one amino acid residue selected from of the group consisting of K14(b), K40(b), K46(b), K49(b), K54(b), K86(b), and K110(b) (these residues having more than 25% of their side chain exposed to the surface), and preferably from the group consisting of K46(b), K54(b), K86(b), and K110(b) (these residues having more than 50% of their side chain exposed to the surface). The FSH-α part of this conjugate can be hFSH-α or any of the modified FSH-α polypeptides described herein.

[0111] In a further embodiment, the conjugate of the invention is one which comprises a modified FSH-α and a modified FSH-β, each of which differ from the corresponding hFSH subunit in the removal of at least one of the above identified lysine residues. For instance, the conjugate of the invention can be one wherein the modified FSH-α and modified FSH-β subunit differ from the corresponding hFSH subunit in at least one of K45(a), K63(a), K75(a), and K91(a) and at least one of K46(b), K54(b), K86(b), and K101(b).

[0112] The removal of any of the above lysine residues is preferably achieved by substitution by any other amino acid residue, in particular by an arginine or a glutamine residue.

[0113] ii) Introduction of lysine residues

[0114] In order to obtain a more extensive conjugation it can be desirable to introduce at least one non-naturally occurring lysine residue in hFSH, in particular in a position occupied by an amino acid residue having a side chain which is more than 25% surface exposed and which is not part of a cystine or located at a receptor binding site.

[0115] Accordingly, in a further embodiment, the conjugate of the invention is one which comprises a modified FSH-α having an amino acid residue which differs from that of hFSH-α in the introduction of at least one lysine residue in a position selected from the group consisting of A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), L12(a), Q13(a), E14(a), P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), P24(a), L26(a), M29(a), F33(a), R42(a), S43(a), T46(a), L48(a), V49(a), Q50(a), N52(a), V61(a), S64(a), Y65(a), N66(a), R67(a), V68(a), T69(a), M71(a), G72(a), G73(a), F74(a), N78(a), T80(a), A81(a), H83(a), S85(a), T86(a), Y88(a), Y89(a), H90(a), and S92(a), in particular selected from of the group consisting of A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), Q13(a), E14(a), P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), T46(a), L48(a), V49(a), Q50(a), N52(a), S64(a), N66(a), R67(a), T69(a), G72(a), G73(a), T86(a), Y89(a), H90(a), and S92(a) (these residues having more than 50% of their side chain exposed to the surface), and most preferably in the position R42(a) and/or R67(a), such as R67(a). The FSH-β part of this conjugate can be hFSH-β or any of the modified FSH-β polypeptides described herein.

[0116] In a further embodiment, the conjugate of the invention is one which comprises a modified FSH-β having an amino acid residue which differs from that of hFSH-β in the introduction of at least one lysine residue in a position selected from the group consisting of N1(b), S2(b), E4(b), L5(b), T6(b), N7(b), I8(b), T9(b), E15(b), E16(b), R18(b), F19(b), I21(b), S22(b), N24(b), Y31(b), Y33(b), R35(b), D36(b), L37(b), Y39(b), D41(b), P42(b), A43(b), R44(b), P45(b), I47(b), E55(b), L56(b), V57(b), Y58(b), E59(b), T60(b), V61(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), Y74(b), T75(b), T80(b), Q81(b), H83(b), G85(b), D88(b), S89(b), D90(b), S91(b), D93(b), T95(b), V96(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), and E111(b), in particular selected from of the group consisting of N1(b), N7(b), T9(b), E15(b), E16(b), R18(b), F19(b), N24(b), Y33(b), D41(b), P42(b), A43(b), R44(b), P45(b), I47(b), E55(b), V57(b), Y58(b), E59(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), T75(b), Q81(b), H83(b), D88(b), S89(b), D90(b), S91(b), T95(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), and E111(b) (these residues having more than 50% of their side chain exposed to the surface), and most preferably selected from the group consisting of R18(b), R35(b), R44(b), R62(b), and R97(b), such R18(b), R44(b), R62(b), and R97(b). The FSH-α part of this conjugate can be hFSH-α or any of the modified FSH-α polypeptides described herein.

[0117] In a further embodiment, the conjugate of the invention is one which comprises a modified FSH-α and a modified FSH-β, each of which differ from the corresponding hFSH subunit in the introduction of a lysine residue, preferably by substitution, in at least one of the above identified positions. For instance, the conjugate of the invention can be one wherein the modified FSH-α and modified FSH-β subunit differ from the corresponding hFSH subunit in that a lysine residue has been introduced in at least one of R42(a) and R67(a), and at least one of R18(b), R35(b), R44(b), R62(b), and R97(b), and more preferably in R67(a), and at least one of R18(b), R44(b), R62(b), R97(b)).

[0118] iii) Introduction and removal of lysine residues

[0119] The conjugate of the invention can comprise at least one introduced lysine residue, in particular any of those described in the section entitled "Introduction of lysine residues," and at least one removed lysine residue, in particular any of those described in the section entitled "Removal of lysine residues."

[0120] Preferably, the conjugate comprises a modified FSH-α and/or a modified FSH-β which differs from the corresponding hFSH-α/β in at least one introduced and at least one removed lysine residue, wherein the lysine residue is introduced by substitution of an amino acid residue selected from the group consisting of R42(a) and R67(a), R18(b), R35(b), R44(b), R62(b), and R97(b), and more preferably from the group consisting of R67(a), R18(b), R44(b), R62(b), and R97(b) and removal of a lysine residue selected from the group consisting of K45(a), K63(a), K75(a), K91(a) K46(b), K54(b), K86(b), and K110(b), the removal preferably being achieved by substitution by any other amino acid residue, in particular by an arginine residue.

[0121] N-terminal Pegylation of FSH

[0122] As indicated above, one aspect of the invention relates to a polypeptide conjugate wherein at least one of the FSH-α and FSH-β subunits comprises a polymer molecule bound to the N-terminal thereof. Preferably, the polymer is a polyethylene glycol (PEG) such as mPEG; see the general discussion below regarding conjugates comprising polyethylene glycol-derived polymers.

[0123] In the case of N-terminal PEGylated FSH conjugates according to the invention, the respective subunits can comprise one or more of the modifications disclosed elsewhere herein, or one or both of the subunits can be the respective wildtype subunits with a PEG-derived polymer being attached at the N-terminal. Thus, the polypeptide conjugate can be one in which the FSH-α subunit comprises hFSH-α having the sequence shown in SEQ ID NO:2, and/or in which the FSH-β subunit comprises hFSH-β having the sequence shown in SEQ ID NO:4. In one embodiment, both of the subunits correspond to the respective wildtype hFSH subunits, although with either the α or β subunit, or both, being N-terminally PEGylated. In a preferred embodiment, however, at least one glycosylation site has been introduced into one or both of the subunits as described in detail above. In cases where at least one of the subunits has an N-terminally attached PEG molecule, it will often be desirable that no other PEG molecules are attached, e.g., to a lysine residue. In such cases, the polypeptide conjugate will thus comprise either one or two N-terminally attached PEG molecules as the sole polymer molecule(s).

[0124] Aldehyde-activated PEG and reduction using NaBH₃CN have been used to selectively pegylate the N-terminal α-amino group of proteins (see for instance U.S. Pat. No. 5,824,784 regarding N-terminal PEGylation of G-CSF). The N-terminus of the α and/or the β chain of wildtype FSH or a modified form of FSH can be PEGylated using similar methods. Reaction materials include purified FSH or a modified form of FSH, methoxy PEG aldehyde (M PEG CHO), and NaBH₃CN. In order to optimise yield, one can for instance vary: molar ratio of FSH, M-PEG-CHO and NaBH₃CN, time for establishment of the Schiff's base

equilibrium (reaction between FSH and M-PEG-CHO before addition of NaBH₃CN), reaction time after addition of NaBH₃CN, temperature, pH, or reaction volume. The yield of PEGylated FSH forms can be analysed using Western blotting, mass spectrometry and N-terminal sequencing. In order to restrict PEGylation to only one of the two N-termini in FSH, PEGylation of the α or β chain can be selectively prevented by addition of a glutamine to the N-terminus. Spontaneous cyclisation of such an N-terminal glutamine residue will render it unaccessible for PEGylation. Such a glutamine residue can subsequently be removed using a pyroglutamyl aminopeptidase (for instance EC 3.4.19.3).

[0125] Conjugate of the Invention Having a Non-lysine Residue as an Attachment Group

[0126] Based on the present disclosure the skilled person will be aware that amino acid residues comprising other attachment groups can be introduced into and/or removed from FSH-α and/or FSH-β, using the same approach as that illustrated above by lysine residues. For instance, one or more amino acid residues comprising an acid group (glutamic acid or aspartic acid), asparagine, tyrosine or cysteine can be introduced into positions which in hFSH are occupied by amino acid residues having surface exposed side chains (i.e., the positions mentioned above as being of interest for introduction of lysine residues), or removed. As described above, introduction or removal of such amino acid residues is preferably performed by substitution. Preferably, Asp is substituted by Asn, Glu by Gln, Tyr by Phe, and Cys by Ser. Another possibility is introduction and/or removal of a histidine, e.g., by substitution with arginine.

[0127] Non-polypeptide Moiety of the Conjugate of the Invention

[0128] As indicated above, the non-polypeptide moiety of the conjugate of the invention is preferably selected from the group consisting of a polymer molecule, a lipophilic compound, an oligosaccharide moiety (by way of in vivo glycosylation) and an organic derivatizing agent. All of these agents can confer desirable properties to the polypeptide part of the conjugate, in particular an increased functional in vivo half-life and/or an increased serum half-life. The polypeptide part of the conjugate is often conjugated to only one type of non-polypeptide moiety, but can also be conjugated to two or more different types of non-polypeptide moieties, e.g., to a polymer molecule and an oligosaccharide moiety, to a lipophilic group and an oligosaccharide moiety, to an organic derivatizing agent and an oligosaccharide moiety, to a lipophilic group and a polymer molecule, etc. The conjugation to two or more different non-polypeptide moieties can be done simultaneously or sequentially. In a preferred embodiment of a polypeptide conjugated to different types of non-polypeptide moieties, the polypeptide is conjugated to one or more oligosaccharide moieties by in vivo glycosylation, and to one or more polymer molecules, preferably PEG, more preferably at an N-terminal, by conjugation in vitro.

[0129] Polypeptide of the Invention

[0130] In a further aspect, the invention relates to a modified FSH-α or a modified FSH-β polypeptide constituting part of a conjugate of the invention. The modified FSH-α and FSH-β are preferably glycosylated and thus

further comprise N-linked and/or O-linked oligosaccharide moieties. Specific modified FSH-α and FSH-β polypeptides of the invention are those described in the section entitled "Conjugate of the invention."

[0131] Methods of Preparing a Conjugate of the Invention

[0132] In the following sections "Conjugation to an oligosaccharide moiety,""Conjugation to a polymer molecule,""Conjugation to a lipophilic compound" and "Conjugation to an organic derivatizing agent," conjugation to specific types of non-polypeptide moieties is described.

[0133] Coupling to an oligosaccharide moiety

[0134] For in vivo glycolyslation, conjugation to an oligosaccharide moiety takes place by means of a glycosylating, eucaryotic expression host. The expression host cell can be selected from fungal (filamentous fungal or yeast), insect or animal cells or from transgenic plant cells. In one embodiment, the host cell is a mammalian cell, such as a CHO cell, e.g., CHO K1, a BHK or HEK cell, e.g., HEK 293, an insect cell such as an SF9 cell, or a yeast cell, e.g., S. cerevisiae or Pichia pastoris, or any of the host cells mentioned hereinafter. Preferred cells for expression of an in vivo glycosylated protein of the invention are mammalian cells, in particular CHO cells.

[0135] Conjugation to a polymer molecule

[0136] The polymer molecule to be coupled to the polypeptide can be any suitable polymer molecule, such as a natural or synthetic homo-polymer or hetero-polymer, typically with a molecular weight in the range of 300-50,000 Da, such as 500-20,000 Da, more preferably in the range of 1000-15,000 Da, such as in the range of 1000-12,000 Da or 2000-10,000 Da. Examples of homo-polymers include a polyol (i.e., poly-OH), a polyamine (i.e., poly-NH₂) and a polycarboxylic acid (i.e., poly-COOH). A hetero-polymer is a polymer which comprises different coupling groups, such as a hydroxyl group and an amine group.

[0137] Examples of suitable polymer molecules include polymer molecules selected from the group consisting of polyalkylene oxide (PAO), including polyalkylene glycol (PAG), such as polyethylene glycol (PEG) and polypropylene glycol (PPG), branched PEGs, poly-vinyl alcohol (PVA), poly-carboxylate, poly-(vinylpyrolidone), polyethylene-co-maleic acid anhydride, polystyrene-co-maleic acid anhydride, dextran, including carboxymethyl-dextran, or any other biopolymer suitable for reducing immunogenicity and/or increasing functional in vivo half-life and/or serum half-life. Another example of a polymer molecule is human albumin or another abundant plasma protein. Generally, polyalkylene glycol-derived polymers are biocompatible, non-toxic, non-antigenic, non-immunogenic, have various water solubility properties, and are easily excreted from living organisms.

[0138] PEG is the preferred polymer molecule, since it has only few reactive groups capable of cross-linking compared to e.g., polysaccharides such as dextran. In particular, monofunctional PEG, e.g., methoxypolyethylene glycol (mPEG), is of interest since its coupling chemistry is relatively simple (only one reactive group is available for conjugating with attachment groups on the polypeptide). Consequently, the risk of cross-linking is eliminated, the resulting polypeptide conjugates are more homogeneous and the reaction of the polymer molecules with the polypeptide is easier to control.

[0139] To effect covalent attachment of the polymer molecule(s) to the polypeptide, the hydroxyl end groups of the polymer molecule must be provided in activated form, i.e., with reactive functional groups. Suitable activated polymer molecules are commercially available, e.g., from Shearwater Polymers, Inc., Huntsville, Ala., USA, or from PolyMASC Pharmaceuticals plc, UK. Alternatively, the polymer molecules can be activated by conventional methods known in the art, e.g., as disclosed in WO 90/13540. Specific examples of activated linear or branched polymer molecules for use in the present invention are described in the Shearwater Polymers, Inc. 1997 and 2000 Catalogs (Functionalized Biocompatible Polymers for Research and Pharmaceuticals, Polyethylene Glycol and Derivatives, incorporated herein by reference). Specific examples of activated PEG polymers include the following linear PEGs: NHS-PEG (e.g., SPA-PEG, SSPA-PEG, SBA-PEG, SS-PEG, SSA-PEG, SC-PEG, SG-PEG, and SCM-PEG), and NOR-PEG), BTC-PEG, EPOX-PEG, NCO-PEG, NPC-PEG, CDI-PEG, ALD-PEG, TRES-PEG, VS-PEG, IODO-PEG, and MAL-PEG, and branched PEGs such as PEG2-NHS and those disclosed in U.S. Pat. Nos. 5,932,462 and 5,643,575, both of which are incorporated herein by reference. Furthermore, the following publications, incorporated herein by reference, disclose useful polymer molecules and/or PEGylation chemistries: U.S. Pat. Nos. 5,824,778, 5,476,653, WO 97/32607, EP 229,108, EP 402,378, U.S. Pat. Nos. 4,902,502, 5,281, 698, 5,122,614, 5,219,564, WO 92/16555, WO 94/04193, WO 94/14758, WO 94/17039, WO 94/18247, WO 94/28024, WO 95/00162, WO 95/11924, WO95/13090, WO 95/33490, WO 96/00080, WO 97/18832, WO 98/41562, WO 98/48837, WO 99/32134, WO 99/32139, WO 99/32140, WO 96/40791, WO 98/32466, WO 95/06058, EP 439 508, WO 97/03106, WO 96/21469, WO 95/13312, EP 921 131, U.S Pat. No. 5,736,625, WO 98/05363, EP 809 996, U.S. Pat. No. 5,629,384, WO 96/41813, WO 96/07670, U.S. Pat. Nos. 5,473,034, 5,516,673, EP 605 963, U.S. Pat. No. 5,382,657, EP 510 356, EP 400 472, EP 183 503 and EP 154 316.

[0140] The conjugation of the polypeptide and the activated polymer molecules is conducted by use of any conventional method, e.g., as described in the following references (which also describe suitable methods for activation of polymer molecules): R. F. Taylor, (1991), "Protein immobilisation. Fundamental and applications," Marcel Dekker, N. Y.; S. S. Wong, (1992), "Chemistry of Protein Conjugation and Crosslinking," CRC Press, Boca Raton; G. T. Hermanson et al., (1993), "Immobilized Affinity Ligand Techniques," Academic Press, N.Y.). The skilled person will be aware that the activation method and/or conjugation chemistry to be used depends on the attachment group(s) of the polypeptide (examples of which are given further above), as well as the functional groups of the polymer (e.g., being amine, hydroxyl, carboxyl, aldehyde, sulfydryl, succinimidyl, maleimide, vinysulfone or haloacetate). The PEGylation can be directed towards conjugation to all available attachment groups on the polypeptide (i.e., such attachment groups that are exposed at the surface of the polypeptide) or can be directed towards one or more specific attachment groups, e.g., the N-terminal amino group (U.S. Pat. No. 5,985,265). Furthermore, the conjugation can be achieved in one step or in a stepwise manner (e.g., as described in WO 99/55377).

[0141] It will be understood that the PEGylation is designed so as to produce the optimal molecule with respect to the number of PEG molecules attached, the size and form of such molecules (e.g., whether they are linear or branched), and where in the polypeptide such molecules are attached. The molecular weight of the polymer to be used will be chosen taking into consideration the desired effect to be achieved. For instance, if the primary purpose of the conjugation is to achieve a conjugate having a high molecular weight and larger size (e.g., to reduce renal clearance), one can choose to conjugate either one or a few high molecular weight polymer molecules or a number of polymer molecules with a smaller molecular weight to obtain the desired effect. For epitope shielding, a sufficiently high number (e.g., 2-8, such as 3-6) of low molecular weight polymer molecules (e.g., with a molecular weight of about 5,000 Da) can be used to effectively shield all or most epitopes of the polypeptide.

[0142] When the protein is conjugated to only a single polymer molecule, for example where an N-terminal PEG molecule is the only polymer molecule, it will often be advantageous that the polymer molecule, which can be linear or branched, has a relatively high molecular weight, e.g., about 12-20 kDa.

[0143] In a specific embodiment, the polypeptide conjugate of the invention comprises a PEG molecule attached to most or substantially all of the lysine residues in the polypeptide available for PEGylation, in particular a linear or branched PEG molecule, e.g., with a molecular weight of about 5 kDa. In this case, it will normally be desirable to remove one or more of the lysines present in wildtype hFSH-α or hFSH-β in order to provide a more limited number of attachment sites and obtain a desired distribution of the PEG molecules. The polypeptide conjugate can further comprise a PEG molecule attached to the N-terminal amino acid residue in addition to the lysine residues.

[0144] Normally, the polymer conjugation is performed under conditions aiming at reacting as many of the available polymer attachment groups as possible with polymer molecules. This is achieved by means of a suitable molar excess of the polymer in relation to the polypeptide. Typical molar ratios of activated polymer molecules to polypeptide are up to about 1000-1, such as up to about 200-1 or up to about 100-1. In some cases, the ratio can be somewhat lower, however, such as up to about 50-1, 10-1 or 5-1.

[0145] It is also contemplated according to the invention to couple the polymer molecules to the polypeptide through a linker. Suitable linkers are well known to the skilled person. A preferred example is cyanuric chloride (Abuchowski et al., (1977), J. Biol. Chem., 252, 3578-3581; U.S. Pat. No. 4,179,337; Shafer et al., (1986), J. Polym. Sci. Polym. Chem. 24, 375-378.

[0146] Subsequent to the conjugation residual activated polymer molecules are blocked according to methods known in the art, e.g., by addition of primary amine to the reaction mixture, and the resulting inactivated polymer molecules removed by a suitable method.

[0147] Covalent in vitro coupling of carbohydrate moieties glycosides (such as dextran) to amino acid residues of the polypeptide can also be used, e.g., as described in WO 87/05330 and in Aplin et al., CRC Crit Rev. Biochem., pp.

259-306, 1981. The in vitro coupling of carbohydrate moieties or PEG to protein- and peptide-bound Gln-residues can be carried out by transglutaminases (TGases). Transglutaminases catalyse the transfer of donor amine groups to protein- and peptide-bound Gln residues in a so-called cross-linking reaction. The donor amine groups can be protein- or peptide-bound e.g., as the ε-amino group in Lys residues or can be part of a small or large organic molecule. An example of a small organic molecule functioning as amino-donor in TGase-catalysed cross-linking is putrescine (1,4-diaminobutane). An example of a larger organic molecule functioning as amino-donor in TGase-catalysed cross-linking is an amine-containing PEG (Sato et al., *Biochemistry* 35, 13072-13080).

[0148] TGases, in general, are highly specific enzymes, and not every Gln residue exposed on the surface of a protein is accessible to TGase-catalysed cross-linking to amino-containing substances. On the contrary, only a few Gln residues function naturally as TGase substrates but the exact parameters governing which Gln residues are good TGase substrates remain unknown. Thus, in order to render a protein susceptible to TGase-catalysed cross-linking reactions it is often a prerequisite at convenient positions to add stretches of amino acid sequence known to function very well as TGase substrates. Several amino acid sequences are known to be or to contain excellent natural TGase substrates e.g., substance P, elafin, fibrinogen, fibronectin, α_2-plasmin inhibitor, α-caseins, and β-caseins.

[0149] Conjugation to a lipophilic compound

[0150] The polypeptide and the lipophilic compound can be conjugated to each other either directly or by use of a linker. The lipophilic compound can be a natural compound such as a saturated or unsaturated fatty acid, a fatty acid diketone, a terpene, a prostaglandin, a vitamin, a carotenoid or steroid, or a synthetic compound such as a carbon acid, an alcohol, an amine and sulphonic acid with one or more alkyl, aryl, alkenyl or other multiple unsaturated compounds. The conjugation between the polypeptide and the lipophilic compound, optionally through a linker, can be done according to methods known in the art, e.g., as described by Bodanszky in Peptide Synthesis, John Wiley, New York, 1976 and in WO 96/12505.

[0151] Coupling to an organic derivatizing agent

[0152] Covalent modification of the polypeptide exhibiting FSH activity can be performed by reacting one or more attachment groups of the polypeptide with an organic derivatizing agent. Suitable derivatizing agents and methods are well known in the art. For example, cysteinyl residues most commonly are reacted with α-haloacetates (and corresponding amines), such as chloroacetic acid or chloroacetamide, to give carboxymethyl or carboxyamidomethyl derivatives. Cysteinyl residues also are derivatized by reaction with bromotrifluoroacetone, α-bromo-β-(4-imidozoyl-)propionic acid, chloroacetyl phosphate, N-alkylmaleimides, 3-nitro-2-pyridyl disulfide, methyl 2-pyridyl disulfide, p-chloromercuribenzoate, 2-chloromercuri-4-nitrophenol, or chloro-7-nitrobenzo-2-oxa-1,3-diazole. Histidyl residues are derivatized by reaction with diethylpyrocarbonate at, pH 5.5-7.0, because this agent is relatively specific for the histidyl side chain. Para-bromophenacyl bromide is also useful. The reaction is preferably performed in 0.1 M sodium cacodylate at pH 6.0. Lysinyl and amino terminal

residues are reacted with succinic or other carboxylic acid anhydrides. Derivatization with these agents has the effect of reversing the charge of the lysinyl residues. Other suitable reagents for derivatizing α-amino-containing residues include imidoesters such as methyl picolinimidate, pyridoxal phosphate, pyridoxal, chloroborohydride, trinitrobenzenesulfonic acid, O-methylisourca, 2,4-pentanedione and transaminase-catalyzed reaction with glyoxylate. Arginyl residues are modified by reaction with one or several conventional reagents, among them phenylglyoxal, 2,3-butanedione, 1,2-cyclohexanedione, and ninhydrin. Derivatization of arginine residues requires that the reaction be performed under alkaline conditions because of the high pKa of the guanidine functional group.

[0153] Furthermore, these reagents can react with the groups of lysine as well as the arginine guanidino group. Carboxyl side groups (aspartyl or glutamyl) are selectively modified by reaction with carbodiimides (R—N=C=N—R'), where R and R' are different alkyl groups, such as 1-cyclohexyl-3-(2-morpholinyl-4-ethyl) carbodiimide or 1-ethyl-3-(4-azonia-4,4-dimethyl-pentyl) carbodiimide. Furthermore, aspartyl and glutamyl residues are converted to asparaginyl and glutaminyl residues by reaction with ammonium ions.

[0154] Blocking of a functional site

[0155] It has been reported that excessive polymer conjugation can lead to a loss of activity of the polypeptide to which the polymer is conjugated. This problem can be eliminated by e.g., removal of attachment groups located at the functional site or by blocking the functional site prior to conjugation. The latter strategy constitutes a further embodiment of the invention (the first strategy being exemplified further above, e.g., by removal of lysine residues which can be located close to the functional site). More specifically, according to the second strategy the conjugation between the polypeptide and the non-polypeptide moiety is conducted under conditions where the functional site of the polypeptide is blocked by a helper molecule capable of binding to the functional site of the polypeptide.

[0156] Preferably, the helper molecule is one which specifically recognizes a functional site of the polypeptide, such as a receptor, in particular the FSH receptor or a part of the FSH receptor. Alternatively, the helper molecule can be an antibody, in particular a monoclonal antibody recognizing the polypeptide exhibiting FSH activity. In particular, the helper molecule can be a neutralizing monoclonal antibody.

[0157] The polypeptide is allowed to interact with the helper molecule before effecting conjugation. This ensures that the functional site of the polypeptide is shielded or protected and consequently unavailable for derivatization by the non-polypeptide moiety such as a polymer. Following its elution from the helper molecule, the conjugate between the non-polypeptide moiety and the polypeptide can be recovered with at least a partially preserved functional site.

[0158] The subsequent conjugation of the polypeptide having a blocked functional site to a polymer, a lipophilic compound, an oligosaccharide moiety, an organic derivatizing agent or any other compound is conducted in the normal way, e.g., as described in the sections above entitled "Conjugation to . . . "

[0159] Irrespective of the nature of the helper molecule to be used to shield the functional site of the polypeptide from conjugation, it is desirable that the helper molecule is free of or comprises only a few attachment groups for the non-polypeptide moiety of choice in any parts of the molecule where the conjugation to such groups will hamper the desorption of the conjugated polypeptide from the helper molecule. Hereby, selective conjugation to attachment groups present in non-shielded parts of the polypeptide can be obtained and it is possible to reuse the helper molecule for repeated cycles of conjugation. For instance, if the non-polypeptide moiety is a polymer molecule such as PEG which has the epsilon amino group of a lysine or N-terminal amino acid residue as an attachment group, it is desirable that the helper molecule is substantially free of conjugatable epsilon amino groups, preferably free of any epsilon amino groups. Accordingly, in a preferred embodiment, the helper molecule is a protein or peptide capable of binding to the functional site of the polypeptide, which protein or peptide is free of any conjugatable attachment groups for the non-polypeptide moiety of choice.

[0160] In a further embodiment, the helper molecule is first covalently linked to a solid phase such as column packing materials, for instance Sephadex or agarose beads, or a surface, e.g., a reaction vessel. Subsequently, the polypeptide is loaded onto the column material carrying the helper molecule and conjugation carried out according to methods known in the art, e.g., as described in the sections above entitled "Conjugation to . . ." This procedure allows the polypeptide conjugate to be separated from the helper molecule by elution. The polypeptide conjugate is eluated by conventional techniques under physico-chemical conditions that do not lead to a substantive degradation of the polypeptide conjugate. The fluid phase containing the polypeptide conjugate is separated from the solid phase to which the helper molecule remains covalently linked. The separation can be achieved in other ways: For instance, the helper molecule can be derivatised with a second molecule (e.g.,

helper molecule-second molecule complex, but not the polypeptide conjugate. The polypeptide conjugate can be released from the helper molecule in any appropriate fashion. Deprotection can be achieved by providing conditions in which the helper molecule dissociates from the functional site of the FSH to which it is bound. For instance, a complex between an antibody to which a polymer is conjugated and an anti-idiotypic antibody can be dissociated by adjusting the pH to an acid or alkaline pH.

[0161] Conjugation of a tagged polypeptide

[0162] In an alternative embodiment, the polypeptide is expressed as a fusion protein with a tag, i.e., an amino acid sequence or peptide stretch made up of typically 1-30, such as 1-20 amino acid residues. Besides allowing for fast and easy purification, the tag is a convenient tool for achieving conjugation between the tagged polypeptide and the non-polypeptide moiety. In particular, the tag can be used for achieving conjugation in microtiter plates or other carriers, such as paramagnetic beads, to which the tagged polypeptide can be immobilised via the tag. The conjugation to the tagged polypeptide in, e.g., microtiter plates has the advantage that the tagged polypeptide can be immobilised in the microtiter plates directly from the culture broth (in principle without any purification) and subjected to conjugation. Thereby, the total number of process steps (from expression to conjugation) can be reduced. Furthermore, the tag can function as a spacer molecule ensuring an improved accessibility to the immobilised polypeptide to be conjugated. The conjugation using a tagged polypeptide can be to any of the non-polypeptide moieties disclosed herein, e.g., to a polymer molecule such as PEG.

[0163] The identity of the specific tag to be used is not critical as long as the tag is capable of being expressed with the polypeptide and is capable of being immobilised on a suitable surface or carrier material. A number of suitable tags are commercially available, e.g., from Unizyme Laboratories, Denmark. For instance, the tag can consist of any of the following sequences:

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His-His-His-His-His-His

Met-Lys-His-His-His-His-His-His

Met-Lys-His-His-Ala-His-His-Gln-His-His

Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln

Met-Lys-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-His-Gln-Gln

or any of the following:
EQKLI SEEDL (a C-terminal tag described in Mol. Cell. Biol. 5:3610-16, 1985)

DYKDDDDK (a C- or N-terminal tag)

YPYDVPDYA
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biotin) that can be recognized by a specific binder (e.g., streptavidin). The specific binder can be linked to a solid phase thereby allowing the separation of the polypeptide conjugate from the helper molecule-second molecule complex through passage over a second helper-solid phase column which will retain, upon subsequent elution, the

[0164] Antibodies against the above tags are commercially available, e.g., from ADI, Aves Lab and Research Diagnostics.

[0165] The subsequent cleavage of the tag from the polypeptide can be achieved by use of commercially available enzymes.

[0166] Methods for Preparing a Polypeptide of the Invention or the Polypeptide of the Conjugate of the Invention

[0167] The polypeptide of the present invention or the polypeptide part of a conjugate of the invention, optionally in glycosylated form, can be produced by any suitable method known in the art. Such methods include constructing a nucleotide sequence encoding the polypeptide and expressing the sequence in a suitable transformed or transfected host. Polypeptides of the invention can also be produced, albeit less efficiently, by chemical synthesis or a combination of chemical synthesis and recombinant DNA technology.

[0168] FSH-α and FSH-β are preferably expressed by the same host cell, thus becoming dimerized in vivo prior to purification and possible in vitro conjugation to a non-polypeptide moiety. Co-expression of FSH-α and FSH-β in CHO cells is e.g., described by Keene et al., *J Biol Chem* 1989 25; 264(9): 4769-75. Alternatively, the polypeptide can be expressed as a single-chain polypeptide wherein the nucleotide sequences encoding FSH-α and FSH-β are fused, either directly or using a suitable peptide linker, and expressed as a single-chain polypeptide using a similar approach to that described in U.S. Pat. No. 5,883,073 or WO 96/05224. It will thus be understood that the polypeptide of the invention can comprise the FSH-α and FSH-β subunits in the form of two separate polypeptide chains, where the two chains become dimerized in vivo so as to form a dimeric polypeptide, or it can comprise a single-chain construct comprising the two subunits covalently linked by a peptide bond or a peptide linker.

[0169] In an alternative embodiment, two FSH-β subunits, wherein at least one of the two β subunits is modified as described herein, preferably by introduction of at least one N- or O-glycosylation site, can be expressed as a single-chain polypeptide in which the subunits are either fused directly or via a peptide linker. Similarly, two FSH-α subunits, wherein at least one of the two α subunits is modified as described herein, can also be expressed as a single-chain polypeptide with the subunits fused directly or via a peptide linker. Further, it is also possible to produce single-chain constructs comprising more than two subunits, e.g., three subunits, wherein at least one of the individual subunits is modified as described herein, and wherein the subunits are fused to each other directly or via a peptide linker. For example, a single-chain construct having the sequence FSHα-FSHβ-FSHβ, FSHβ-FSHα-FSHβ or FSHβ-FSHβ-FSHα, wherein the β subunits in each construct are the identical or different, can be produced using techniques known in the art. Single-chain constructs of this general type are disclosed in U.S. Pat. Nos. 5,705,478, 5,883,073, WO 99/25489 and WO 96/05224

[0170] For single-chain constructs, the linker peptide will often predominantly include the amino acid residues Gly, Ser, Ala and/or Thr. Such a linker typically comprises 1-30 amino acid residues, such as a sequence of about 2-20 or 3-15 amino acid residues. The amino acid residues selected for inclusion in the linker peptide should exhibit properties that do not interfere significantly with the activity of the polypeptide. Thus, the linker peptide should on the whole not exhibit a charge which would be inconsistent with the desired FSH activity, or interfere with internal folding, or form bonds or other interactions with amino acid residues in

one or more of the subunits which would seriously impede the binding of the dimeric or multimeric polypeptide to the receptor.

[0171] Specific linkers for use in the present invention can be designed on the basis of known naturally occurring as well as artificial polypeptide linkers (see, e.g., Hallewell et al. (1989), *J. Biol. Chem.* 264, 5260-5268; Alfthan et al. (1995), *Protein Eng.* 8, 725-731; Robinson & Sauer (1996), *Biochemistry* 35, 109-116; Khandekar et al. (1997), *J. Biol. Chem.* 272, 32190-32197; Fares et al. (1998), *Endocrinology* 139, 2459-2464; Smallshaw et al. (1999), *Protein Eng.* 12, 623-630; U.S. Pat. No. 5,856,456). For instance, linkers used for creating single-chain antibodies, e.g., a 15mer consisting of three repeats of a Gly-Gly-Gly-Gly-Ser amino acid sequence ((Gly$_4$Ser)$_3$), are contemplated to be useful. Furthermore, phage display technology as well as selective infective phage technology can be used to diversify and select appropriate linker sequences (Tang et al., *J. Biol. Chem.* 271, 15682-15686, 1996; Hennecke et al. (1998), *Protein Eng.* 11, 405-410). Also, Arc repressor phage display has been used to optimize the linker length and composition for increased stability of a single-chain protein (Robinson and Sauer (1998), *Proc. Natl. Acad. Sci. USA* 95, 5929-5934).

[0172] Another way of obtaining a suitable linker is by optimizing a simple linker, e.g., ((Gly$_4$Ser)$_n$), through random mutagenesis. The linker can e.g., be (Gly$_4$Ser)$_n$ or (Gly$_3$Ser)$_n$ where n is 1, 2, 3 or 4.

[0173] The nucleotide sequence encoding FSH-α or FSH-β modified according to the invention can be constructed by isolating or synthesizing a nucleotide sequence encoding the parent FSH subunit, such as hFSH-α or hFSH-β with the amino acid sequence shown in SEQ ID NO:2 or 4, respectively, or the precursor form thereof (shown in SEQ ID NO:1 and 3, respectively) and then changing the nucleotide sequence so as to effect introduction (i.e., insertion or substitution) or deletion (i.e., removal or substitution) of the relevant amino acid residue(s). The nucleotide sequence is conveniently modified by site-directed mutagenesis in accordance with conventional methods. Alternatively, the nucleotide sequence can be prepared by chemical synthesis, e.g., by using an oligonucleotide synthesizer, wherein oligonucleotides are designed based on the amino acid sequence of the desired polypeptide, and preferably selecting those codons that are favored in the host cell in which the recombinant polypeptide will be produced. For example, several small oligonucleotides coding for portions of the desired polypeptide can be synthesized and assembled by PCR, ligation or ligation chain reaction (LCR) (Barany, *PNAS* 88:189-193, 1991). The individual oligonucleotides typically contain 5' or 3' overhangs for complementary assembly.

[0174] Once assembled (by synthesis, site-directed mutagenesis or another method), the nucleotide sequence encoding the polypeptide is inserted into a recombinant vector and operably linked to control sequences necessary for expression of the FSH in the desired transformed host cell.

[0175] It should of course be understood that not all vectors and expression control sequences function equally well to express the nucleotide sequence encoding a polypeptide described herein. Neither will all hosts function equally

well with the same expression system. However, one of skill in the art can make a selection among these vectors, expression control sequences and hosts without undue experimentation. For example, in selecting a vector, the host must be considered because the vector must replicate in it or be able to integrate into the chromosome. The vector's copy number, the ability to control that copy number, and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered. In selecting an expression control sequence, a variety of factors should also be considered. These include, for example, the relative strength of the sequence, its controllability, and its compatibility with the nucleotide sequence encoding the polypeptide, particularly as regards potential secondary structures. Hosts should be selected by consideration of their compatibility with the chosen vector, the toxicity of the product coded for by the nucleotide sequence, their secretion characteristics, their ability to fold the polypeptide correctly, their fermentation or culture requirements, and the ease of purification of the products coded for by the nucleotide sequence.

[0176] The recombinant vector can be an autonomously replicating vector, i.e., a vector which exists as an extrachromosomal entity, the replication of which is independent of chromosomal replication, e.g., a plasmid. Alternatively, the vector is one which, when introduced into a host cell, is integrated into the host cell genome and replicated together with the chromosome(s) into which it has been integrated.

[0177] The vector is preferably an expression vector in which the nucleotide sequence encoding the polypeptide of the invention is operably linked to additional segments required for transcription of the nucleotide sequence. The vector is typically derived from plasmid or viral DNA. A number of suitable expression vectors for expression in the host cells mentioned herein are commercially available or described in the literature. Useful expression vectors for mammalian eukaryotic hosts include, for example, vectors comprising expression control sequences from SV40, bovine papilloma virus, adenovirus and cytomegalovirus. Specific vectors are, e.g., pCDNA3.1(+)\Hyg (Invitrogen, Carlsbad, Calif., USA) and pCI-neo (Stratagene, La Jolla, Calif., USA). Useful expression vectors for yeast cells include the 2μ plasmid and derivatives thereof, the POT1 vector (U.S. Pat. No. 4,931,373), the pJSO37 vector described in Okkels, *Ann. New York Acad. Sci.* 782, 202-207, 1996, and pPICZ A, B or C (Invitrogen). Useful vectors for insect cells include pVL941, pBG311 (Cate et al., *Cell* 45, pp. 685-98 (1986)), pBluebac 4.5 and pMelbac (both available from Invitrogen). Useful expression vectors for bacterial hosts include known bacterial plasmids, such as plasmids from *E. coli*, including pBR322, pET3a and pET12a (both from Novagen Inc., WI, USA), wider host range plasmids, such as RP4, phage DNAs, e.g., the numerous derivatives of phage lambda, e.g., NM989, and other DNA phages, such as M13 and filamentous single stranded DNA phages.

[0178] Other vectors for use in this invention include those that allow the nucleotide sequence encoding the polypeptide to be amplified in copy number. Such amplifiable vectors are well known in the art. They include, for example, vectors able to be amplified by DHFR amplification (see, e.g., Kaufman, U.S. Pat. No. 4,470,461, Kaufman and Sharp,

Mol. Cell. Biol. 2, pp. 1304-19 (1982)) and glutamine synthetase ("GS") amplification (see, e.g., U.S. Pat. No. 5,122,464 and EP 338,841).

[0179] In one embodiment, a pair of expression vectors are used for expressing the polypeptide subunits of the invention. Each of the vectors of said pair is capable of transfecting a eukaryotic cell as described herein, and the vectors comprise nucleotide sequences encoding, respectively, a modified FSH-α as described herein and a wildtype FSH-β subunit, a modified FSH-β as described herein and a wildtype FSH-α subunit, or a modified FSH-α and a modified FSH-β as described herein. The use of a pair of vectors is e.g., described in EP 211,894. Alternatively, a single expression vector comprising nucleotide sequences encoding both the FSH-α subunit and the FSH-β subunit, where at least one of the subunits is modified as described herein, can be used for expressing the polypeptide subunits.

[0180] The recombinant vector can further comprise a DNA sequence enabling the vector to replicate in the host cell in question. An example of such a sequence (when the host cell is a mammalian cell) is the SV40 origin of replication. When the host cell is a yeast cell, suitable sequences enabling the vector to replicate are the yeast plasmid 2μ replication genes REP 1-3 and origin of replication.

[0181] The vector can also comprise a selectable marker, e.g., a gene whose product complements a defect in the host cell, such as the gene coding for dihydrofolate reductase (DHFR) or the *Schizosaccharomyces pombe* TPI gene (described by P. R. Russell, Gene 40, 1985, pp. 125-130), or one which confers resistance to a drug, e.g., ampicillin, kanamycin, tetracyclin, chloramphenicol, neomycin, hygromycin or methotrexate. For *Saccharomyces cerevisiae*, selectable markers include ura3 and leu2. For filamentous fungi, selectable markers include amdS, pyrG, arcB, niaD and sC.

[0182] The term "control sequences" is defined herein to include all components which are necessary or advantageous for the expression of the polypeptide of the invention. Each control sequence can be native or foreign to the nucleic acid sequence encoding the polypeptide. Such control sequences include, but are not limited to, a leader sequence, polyadenylation sequence, propeptide sequence, promoter, enhancer or upstream activating sequence, signal peptide sequence, and transcription terminator. At a minimum, the control sequences include a promoter.

[0183] A wide variety of expression control sequences can be used in the present invention. Such useful expression control sequences include the expression control sequences associated with structural genes of the foregoing expression vectors as well as any sequence known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof.

[0184] Examples of suitable control sequences for directing transcription in mammalian cells include the early and late promoters of SV40 and adenovirus, e.g., the adenovirus 2 major late promoter, the MT-1 (metallothionein gene) promoter, the human cytomegalovirus immediate-early gene promoter (CMV), the human elongation factor 1α (EF-1α) promoter, the Drosophila minimal heat shock protein 70 promoter, the Rous Sarcoma Virus (RSV) promoter, the

human ubiquitin C (UbC) promoter, the human growth hormone terminator, SV40 or adenovirus Elb region polyadenylation signals and the Kozak consensus sequence (Kozak, M. J Mol Biol Aug. 20, 1987;196(4):947-50).

[0185] In order to improve expression in mammalian cells a synthetic intron can be inserted in the 5' untranslated region of the nucleotide sequence encoding the polypeptide. An example of a synthetic intron is the synthetic intron from the plasmid pCI-Neo (available from Promega Corporation, WI, USA).

[0186] Examples of suitable control sequences for directing transcription in insect cells include the polyhedrin promoter, the P10 promoter, the *Autographa californica* polyhedrosis virus basic protein promoter, the baculovirus immediate early gene 1 promoter and the baculovirus 39K delayed-early gene promoter, and the SV40 polyadenylation sequence. Examples of suitable control sequences for use in yeast host cells include the promoters of the yeast α-mating system, the yeast triose phosphate isomerase (TPI) promoter, promoters from yeast glycolytic genes or alcohol dehydrogenase genes, the ADH2-4c promoter, and the inducible GAL promoter. Examples of suitable control sequences for use in filamentous fungal host cells include the ADH3 promoter and terminator, a promoter derived from the genes encoding *Aspergillus oryzae* TAKA amylase triose phosphate isomerase or alkaline protease, an *A. niger* α-amylase, *A. niger* or *A. nidulans* glucoamylase, *A. nidulans* acetamidase, *Rhizomucor miehei* aspartic proteinase or lipase, the TPI1 terminator and the ADH3 terminator. Examples of suitable control sequences for use in bacterial host cells include promoters of the lac system, the trp system, the TAC or TRC system, and the major promoter regions of phage lambda.

[0187] The presence or absence of a signal peptide will, e.g., depend on the expression host cell used for the production of the polypeptide to be expressed (whether it is an intracellular or extracellular polypeptide) and whether it is desirable to obtain secretion. For use in filamentous fungi, the signal peptide can conveniently be derived from a gene encoding an Aspergillus sp. amylase or glucoamylase, a gene encoding a *Rhizomucor miehei* lipase or protease or a *Humicola lanuginosa* lipase. The signal peptide is preferably derived from a gene encoding *A. oryzae* TAKA amylase, *A. niger* neutral α-amylase, *A. niger* acid-stable amylase, or *A. niger* glucoamylase. For use in insect cells, the signal peptide can conveniently be derived from an insect gene (cf. WO 90/05783), such as the *Lepidopteran manduca sexta* adipokinetic hormone precursor, (cf. U.S. Pat. No. 5,023,328), the honeybee melittin (Invitrogen), ecdysteroid UDPglucosyltransferase (egt) (Murphy et al., *Protein Expression and Purification* 4, 349-357 (1993) or human pancreatic lipase (hpl) (*Methods in Enzymology* 284, pp. 262-272, 1997). A preferred signal peptide for use in mammalian cells is that of hFSH or the murine Ig kappa light chain signal peptide (Coloma, M (1992) *J. Imm. Methods* 152:89-104). For use in yeast cells suitable signal peptides have been found to be the α-factor signal peptide from *S. cereviciae* (cf. U.S. Pat. No. 4,870,008), a modified carboxypeptidase signal peptide (cf. L. A. Valls et al., *Cell* 48, 1987, pp. 887-897), the yeast BAR1 signal peptide (cf. WO 87/02670), the yeast aspartic protease 3 (YAP3) signal peptide (cf. M. Egel-Mitani et al., *Yeast* 6, 1990, pp. 127-137), and the synthetic leader sequence TA57 (WO98/32867). For use in *E. coli* cells a suitable signal peptide have been found to be the signal peptide ompA (EP581821).

[0188] The nucleotide sequences of the invention encoding the dimeric polypeptide exhibiting FSH activity, whether prepared by site-directed mutagenesis, synthesis, PCR or other methods, can optionally also include a nucleotide sequence that encodes a signal peptide. The signal peptide is present when the polypeptide is to be secreted from the cells in which it is expressed. Such signal peptide, if present, should be one recognized by the cell chosen for expression of the polypeptide. The signal peptide can be homologous (e.g., be that normally associated with a hFSH subunit) or heterologous (i.e., originating from another source than hFSH) to the polypeptide or can be homologous or heterologous to the host cell, i.e., be a signal peptide normally expressed from the host cell or one which is not normally expressed from the host cell. Accordingly, the signal peptide can be prokaryotic, e.g., derived from a bacterium such as *E. coli*, or eukaryotic, e.g., derived from a mammalian, or insect or yeast cell.

[0189] Any suitable host can be used to produce the polypeptide subunits of the invention, including bacteria, fungi (including yeasts), plant, insect, mammal, or other appropriate animal cells or cell lines, as well as transgenic animals or plants. Examples of bacterial host cells include gram-positive bacteria such as strains of Bacillus, e.g., *B. brevis* or *B. subtilis*, or Streptomyces, or gram-negative bacteria, such as Pseudomonas or strains of *E. coli*. The introduction of a vector into a bacterial host cell may, for instance, be effected by protoplast transformation (see, e.g., Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), using competent cells (see, e.g., Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), electroporation (see, e.g., Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or conjugation (see, e.g., Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278). Examples of suitable filamentous fungal host cells include strains of Aspergillus, e.g., *A. oryzae*, *A. niger*, or *A. nidulans*, Fusarium or Trichodenna. Fungal cells can be transformed by a process involving protoplast formation, transformation of the protoplasts, and regeneration of the cell wall in a manner known per se. Suitable procedures for transformation of Aspergillus host cells are described in EP 238 023 and U.S. Pat. No. 5,679, 543. Suitable methods for transforming Fusarium species are described by Malardier et al., 1989, *Gene* 78: 147-156 and WO 96/00787. Examples of suitable yeast host cells include strains of Saccharomyces, e.g., *S. cerevisiae*, Schizosaccharomyces, Klyveromyces, Pichia, such as *P. pastoris* or *P. methanolica*, Hansenula, such as *H. Polymorpha* or Yarrowia. Yeast can be transformed using the procedures described by Becker and Guarente, In Abelson, J. N. and Simon, M. I., editors, *Guide to Yeast Genetics and Molecular Biology*, Methods in Enzymology, Volume 194, pp 182-187, Academic Press, Inc., New York; Ito et al., 1983, *Journal of Bacteriology* 153: 163; Hinnen et al., 1978, *PNAS USA* 75: 1920: and as disclosed by Clontech Laboratories, Inc, Palo Alto, Calif., USA (in the product protocol for the Yeastmaker™ Yeast Transformation System Kit). Examples of suitable insect host cells include a Lepidoptora cell line, such as *Spodoptera frugiperda* (Sf9 or Sf21) or *Trichoplusioa ni* cells (High Five) (U.S. Pat. No. 5,077,214). Transformation of insect cells and production of heterologous polypeptides therein can be performed as described by Invitrogen. Examples of suitable mammalian host cells

include Chinese hamster ovary (CHO) cell lines, (e.g., CHO-K1; ATCC CCL-61), Green Monkey cell lines (COS) (e.g., COS 1 (ATCC CRL-1650), COS 7 (ATCC CRL-1651)); mouse cells (e.g., NS/O), Baby Hamster Kidney (BHK) cell lines (e.g., ATCC CRL-1632 or ATCC CCL-10), and human cells (e.g., HEK 293 (ATCC CRL-1573)), as well as plant cells in tissue culture. Additional suitable cell lines are known in the art and available from public depositories such as the American Type Culture Collection, USA. Methods for introducing exogeneous DNA into mammalian host cells include calcium phosphate-mediated transfection, electroporation, DEAE-dextran mediated transfection, liposome-mediated transfection, viral vectors and the transfection method described by Life Technologies Ltd, Paisley, UK using Lipofectamin 2000. These methods are well known in the art and e.g., described by Ausbel et al. (eds.), 1996, Current Protocols in Molecular Biology, John Wiley & Sons, NY, USA. The cultivation of mammalian cells are conducted according to established methods, e.g., as disclosed in (Animal Cell Biotechnology, Methods and Protocols, Edited by Nigel Jenkins, 1999, Human Press Inc, Totowa, N.J., USA and Harrison M A and Rae I F, General Techniques of Cell Culture, Cambridge University Press 1997).

[0190] In the production methods of the present invention, the cells are cultivated in a nutrient medium suitable for production of the polypeptide using methods known in the art. For example, the cell can be cultivated by shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch, fed-batch, or solid state fermentations) in laboratory or industrial fermenters performed in a suitable medium and under conditions allowing the polypeptide to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art. Suitable media are available from commercial suppliers or can be prepared according to published compositions (e.g., in catalogues of the American Type Culture Collection). If the polypeptide is secreted into the nutrient medium, it can be recovered directly from the medium. If the polypeptide is not secreted, it can be recovered from cell lysates.

[0191] The resulting polypeptide can be recovered by methods known in the art. For example, it can be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray drying, evaporation, or precipitation.

[0192] The polypeptides can be purified by a variety of procedures known in the art including, but not limited to, chromatography (e.g., ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (e.g., preparative isoelectric focusing), differential solubility (e.g., ammonium sulfate precipitation), SDS-PAGE, or extraction (see e.g., *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

[0193] Pharmaceutical Composition of the Invention and its use

[0194] In one aspect, the polypeptide, the conjugate or the pharmaceutical composition according to the invention is used for the manufacture of a medicament for treatment of infertility or diseases associated with insufficient endogenous production of FSH.

[0195] In another aspect, the polypeptide, the conjugate or the pharmaceutical composition according to the invention is used in a method of treating an infertile mammal, in particular a human, comprising administering to the mammal in need thereof such polypeptide, conjugate or pharmaceutical composition.

[0196] The polypeptide exhibiting FSH activity of the invention or the conjugate of the invention is administered at a dose approximately paralleling that employed in therapy with rhFSH such as Gonal-F® and Puregon®. However, due to the increased functional in vivo half-life of the conjugate of the invention, it is contemplated that the product will be administered less frequently and at a dose which provides a comparable effect to that obtained in current therapy. It is thus contemplated that the composition of the invention can be administered at substantially less frequent intervals than currently available treatments, e.g., not more often than once every three days, such as not more than once every four, five, six or seven days. Accordingly, the exact dose to be administered will depend on the circumstances, including the patient to be treated, the cause of infertility if known, the status of the ovaries, the patient's plasma FSH concentration prior to treatment, and the functional in vivo half-life of the product. Normally, in the treatment of infertility the dose should be capable of stimulating follicle maturation, e.g., induce follicles to grow about 2 mm per day during a time period of 8-9 days. For instance, for a product having a functional in vivo half-life of 3-4 days, two doses should be given at least three days apart if a relatively stable plasma concentration is desired. Analogously, for a product having a functional in vivo half-life of about 6 days, one dose would suffice during most of the stimulation period.

[0197] The composition of the invention can be exceedingly advantageous when employed in a step-down protocol, i.e., a protocol where decreasing dosages of FSH are given during the stimulation period, but where use of the composition of the invention, e.g., administered in one or two doses as outlined above, can provide such a slowly decreasing plasma concentration of FSH.

[0198] It will be apparent to those of skill in the art that an effective amount of a conjugate, preparation or composition of the invention depends, inter alia, upon the disease, the dose, the administration schedule, whether the polypeptide or conjugate or composition is administered alone or in conjunction with other therapeutic agents, the serum half-life of the compositions, and the general health of the patient. Typically, an effective dose of the conjugate, preparation or composition of the invention is sufficient to ensure development and maturation of follicles at a rate and to a degree compatible with that obtained using standard rhFSH such as Gonal-F® and Puregon®.

[0199] A further contemplated advantage is that the more stable plasma concentration obtained with a composition of the invention results in a more efficient development and maturation of follicles, which subsequently can enable a higher pregnancy rate.

[0200] The polypeptide or conjugate of the invention is normally administered in a composition including one or more pharmaceutically acceptable carriers or excipients. "Pharmaceutically acceptable" means a carrier or excipient that does not cause any untoward effects in patients to whom it is administered. Such pharmaceutically acceptable carriers

and excipients are well known in the art, and the polypeptide or conjugate of the invention can be formulated into pharmaceutical compositions by well-known methods (see e.g., Remington's Pharmaceutical Sciences, 18th edition, A. R. Gennaro, Ed., Mack Publishing Company (1990); Pharmaceutical Formulation Development of Peptides and Proteins, S. Frokjaer and L. Hovgaard, Eds., Taylor & Francis (2000); and Handbook of Pharmaceutical Excipients, 3rd edition, A. Kibbe, Ed., Pharmaceutical Press (2000)). Pharmaceutically acceptable excipients that can be used in compositions comprising the polypeptide or conjugate of the invention include, for example, buffering agents, stabilizing agents, preservatives, isotonifiers, non-ionic surfactants or detergents ("wetting agents"), antioxidants, bulking agents or fillers, chelating agents and cosolvents.

[0201] The pharmaceutical composition of the polypeptide or conjugate of the invention can be formulated in a variety of forms, including liquids, e.g., ready-to-use solutions or suspensions, gels, lyophilized, or any other suitable form, e.g., powder or crystals suitable for preparing a solution. The preferred form will depend upon the particular indication being treated and will be apparent to one of skill in the art.

[0202] The pharmaceutical composition containing the polypeptide or conjugate of the invention can be administered intravenously, intramuscularly, intraperitoneally, intradermally, subcutaneously, sublingualy, buccally, intranasally, transdermally, by inhalation, or in any other acceptable manner, e.g., using PowderJect® or ProLease® technology or a pen injection system. The preferred mode of administration will depend upon the particular indication being treated and will be apparent to one of skill in the art. In particular, it is advantageous that the composition be administered subcutaneously, since this allows the patient to conduct the administration herself.

[0203] The pharmaceutical composition of the invention can be administered in conjunction with other therapeutic agents. These agents can be incorporated as part of the same pharmaceutical composition or can be administered separately from the polypeptide or conjugate of the invention, either concurrently or in accordance with any other acceptable treatment schedule. In addition, the polypeptide, conjugate or pharmaceutical composition of the invention can be used as an adjunct to other therapies.

[0204] By obtaining a more stable FSH plasma concentration just above the threshold level for follicle growth, the composition of the invention is of particular interest for the treatment of women suffering from anovulation WHO type I, II or III, since only 1-2 mature follicles are desired in these patients.

[0205] Furthermore, the invention relates in other aspects to the use of a composition of the invention in a step-down protocol where a decreasing plasma FSH concentration is obtained using only one or two injections, and preferably only a single injection, to the use of a composition of the invention in a step-up protocol where an increase in FSH concentrations is obtained faster using a lower individual as well as total dosage, and to the use of a composition of the invention in combination with compounds for in vitro maturation (sterol derivatives such as FF-MAS and media containing growth and maturation factors known in the art).

[0206] Mixtures of FSH and LH activities (hMG) are routinely used in the treatment of human infertility. This particular combination therapy can be advantageous because gonadal support of gamete maturation is dependent upon the synergistic actions of both FSH and LH. Current treatment protocols requiring FSH and LH activity utilize urinary extracts from postmenopausal women. The use of these extracts is compromised by several factors, including variability.

[0207] It will in some cases be advantageous to administer the composition of the invention as part of a treatment protocol that also involves LH and/or hCG, for example recombinant LH and/or hCG. This can in particular be useful for treatment of women with low endogenous LH levels. Finally, the composition of the invention can be used, possibly in combination with LH, in the treatment of male infertility, in particular of hypogonadotrophic hypogonadism and oligo- or azoospermia. The more stable plasma concentration obtained with a composition of the invention can lead to a more efficient spermatogenesis. Also, a long lasting effect would be particularly advantageous for such treatment due to the long-term treatment period of about three months.

[0208] The present invention will be further illustrated by the following non-limiting methods and examples.

[0209] Structure Analysis Methods

[0210] Sequence numbering

[0211] The amino acid sequence of hFSH-α is numbered according to the mature sequence shown in SEQ ID NO:2; an (a) suffix herein indicates the α chain. The amino acid sequence of hFSH-β is numbered according to the mature sequence shown in SEQ ID NO:4; a (b) suffix herein indicates the β chain.

[0212] Structures

[0213] Human FSH α is identical to the α chain of Human Chorionic Gonadotropin (HCG) for which two published structures are available: Wu, H., Lustbader, J. W., Liu, Y., Canfield, R. E., Hendrickson, W. A.: Structure 2 pp. 545 (1994) and Lapthorn, A. J., Harris, D. C., Littlejohn, A., Lustbader, J. W., Canfield, R. E., Machin, K. J., Morgan, F. J., Isaacs, N. W.: Nature 369 pp. 455 (1994), both including the β chain of HCG. The β chain of hFSH is 32 percent identical to the amino acid sequence of the structural part of the β chain of HCG (see the sequence alignment of FIG. 1). A series of 50 models of the 3D structure of FSH was built based on the above two available hCG structures and based on the sequence alignment in FIG. 1 using the program Modeller 98 (MSI Inc., 1999). The four N-terminal residues (A1(a), P2(a), D3(a) and V4(a) as well as the three C-terminal residues (H90(a), K91(a) and S92(a)) were not modeled as they are not identified in the HCG structures. All of the HFSH-β chain was modeled, even the part which has no homologous residues in the HCG structures.

[0214] Accessible Surface Area (ASA)

[0215] The computer program Access (B. Lee and F. M. Richards, J. Mol. Biol. 55: 379-400 (1971)) version 2 (®1983 Yale University) was used to compute the accessible surface area (ASA) of the individual atoms in the structure. This method typically uses a probe-size of 1.4 Å and defines the Accessible Surface Area (ASA) as the area formed by the

center of the probe. Prior to this calculation all water molecules and all hydrogen atoms should be removed from the coordinate set, as should other atoms not directly related to the protein.

[0216] Fractional ASA of side chain

[0217] The fractional ASA of the side chain atoms is computed by division of the sum of the ASA of the atoms in the side chain with a value representing the ASA of the side chain atoms of that residue type in an extended Ala-x-Ala tripeptide, see Hubbard, Campbell & Thornton (1991) *J. Mol. Biol.* 220,507-530. For this example the CA atom is regarded as being a part of the side chain of glycine residues but not other residues. The following values are used as standard 100% ASA for the side chain:

Ala	69.23	Å^2	Leu	140.76	Å^2
Arg	200.35	Å^2	Lys	162.50	Å^2
Asn	106.25	Å^2	Met	156.08	Å^2
Asp	102.06	Å^2	Phe	163.90	$\text{Å}^{2^{15}}$
Cys	96.69	Å^2	Pro	119.65	Å^2
Gln	140.58	Å^2	Ser	78.16	Å^2
Glu	134.61	Å^2	Thr	101.67	Å^2
Gly	32.28	Å^2	Trp	210.89	$\text{Å}^{2}20$
His	147.00	Å^2	Tyr	176.61	Å^2
Ile	137.91	Å^2	Val	114.14	Å^2

[0218] Determination of surface exposed residues from structural models:

[0219] Surface accessibility and fractional ASA of side chains were calculated for each of the 50 model structures. The average value over the structural ensemble was used in the following. The N- and C-terminal residues of the FSH-α chain not included in the model are defined as having 100% side chain accessibility.

[0220] The following amino acid residues in hFSH-60 and hFSH-β, respectively, have more than 25% of their side chain exposed to the surface:

[0221] A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), L12(a), Q13(a), E14(a), P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), P24(a), L26(a), M29(a), F33(a), R42(a), S43(a), K44(a), K45(a), T46(a), L48(a), V49(a), Q50(a), N52(a), V61(a), K63(a), S64(a), Y65(a), N66(a), R67(a), V68(a), T69(a), M71(a), G72(a), G73(a), F74(a), K75(a), N78(a), T80(a), A81(a), H83(a), C84(a), S85(a), T86(a), Y88(a), Y89(a), H90(a), K91(a), S92(a), N1(b), S2(b), E4(b), L5(b), T6(b), N7(b), T9(b), I8(b), T9(b), K14(b), E15(b), E16(b), R18(b), F19(b), I21(b), S22(b), N24(b), Y31(b), Y33(b), R35(b), D36(b), L37(b), Y39(b), K40(b), D41(b), P42(b), A43(b), R44(b), P45(b), K46(b), I47(b), K49(b), K54(b), E55(b), L56(b), V57(b), Y58(b), E59(b), T60(b), V61(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), Y74(b), T75(b), T80(b), Q81(b), H83(b), G85(b), K86(b), D88(b), S89(b), D90(b), S91(b), D93(b), T95(b), V96(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), K110(b), and E111(b).

[0222] The following amino acid residues have more than 50% of their side chain exposed to the surface:

[0223] A1(a), P2(a), D3(a), V4(a), Q5(a), D6(a), P8(a), E9(a), T11(a), Q13(a), E14(a), P16(a), F17(a), Q20(a), P21(a), G22(a), A23(a), K45(a), T46(a), L48(a), V49(a), Q50(a), N52(a), K63(a), S64(a), N66(a), R67(a), T69(a),

G72(a), G73(a), K75(a), T86(a), Y89(a), H90(a), K91(a), S92(a), N1(b), N7(b), T9(b), E15(b), E16(b), R18(b), F19(b), N24(b), Y33(b), D41(b), P42(b), A43(b), R44(b), P45(b), K46(b), I47(b), K54(b), E55(b), V57(b), Y58(b), E59(b), R62(b), P64(b), G65(b), A67(b), H68(b), H69(b), D71(b), L73(b), T75(b), Q81(b), H83(b), K86(b), D88(b), S89(b), D90(b), S91(b), T95(b), R97(b), G98(b), L99(b), G100(b), Y103(b), S105(b), F106(b), G107(b), E108(b), M109(b), K110(b), and E111(b).

[0224] Determining distances between atoms

[0225] The distance between atoms is most easily determined using molecular graphics software, e.g., InsightII v. 98.0, MSI Inc.

EXAMPLES

Example 1

[0226] Construction of Plasmids for Expression of FSH

[0227] A gene encoding the human FSH-α subunit was constructed by assembly of synthetic oligonucleotides by PCR using methods similar to the ones described in Stemmer et al. (1995) *Gene* 164, pp. 49-53. The native FSH-α signal sequence was maintained in order to allow secretion of the gene product. The codon usage of the gene was optimised for high expression in mammalian cells. Furthermore, in order to achieve high gene expression, an intron (from pCI-Neo (Promega)) was included in the 5' untranslated region of the gene. The synthetic gene was subcloned behind the CMV promoter in pcDNA3.1/Hygro (Invitrogen). The sequence of the resulting plasmid, termed pBvdH977, is given in SEQ ID NO:5 (FSH-α-coding sequence at position 1225 to 1572). Similarly, a synthetic gene encoding the wildtype human FSH-β subunit was constructed. Also in this construct, the native signal sequence was maintained in order to allow secretion, and the codon usage was optimised for high expression and an intron was included in the recipient vector (pcDNA3.1/Zeo (Invitrogen)). The sequence of the resulting FSH-β-containing plasmid, termed pBvdH1022, is given in SEQ ID NO:6 (FSH-β-coding sequence at position 1231 to 1617). A plasmid containing both the FSH-α and the FSH-β encoding synthetic genes was generated by subcloning the FSH-α containing NruI-PvuII fragment from pBvdH977 into pBvdH1022 linearized with NruI. The resulting plasmid, in which the FSH-α and FSH-β-expression cassettes are in direct orientation, was termed pBvdHII1100.

Example 2

[0228] Expression of FSH in CHO Cells

[0229] FSH was expressed in Chinese Hamster Ovary (CHO) K1 cells, obtained from the American Type Culture Collection (ATCC, CCL 61).

[0230] For transient expression of FSH, cells were grown to 95% confluency in serum-containing media (MEMα with ribonucleotides and deoxyribonucleotides (Gibco/BRL Cat # 32571-028) containing 1:10 FBS (BioWhittaker Cat # 02-701F) and 1:100 penicillin and streptomycin (BioWhittaker Cat # BE17-602E), or Dulbecco's MEM/Nut.-mix F-12 (Ham) L-glutamine, 15 mM Hepes, pyridoxine-HCl (Life Technologies Cat # 11039-021) with the same additives. FSH-encoding plasmids were transfected into the cells

using Lipofectamine 2000 (Life Technologies) according to the manufacturer's specifications. 24-48 hrs after transfection, culture media were collected, centrifuged and filtered through 0.22 μm filters to remove cells.

[0231] Stable clones expressing FSH were generated by transfection of CHO K1 cells with FSH-encoding plasmids followed by incubation of the cells in selective media (for instance one of the above media containing 0.5 mg/ml zeocin for cells transfected with plasmid pBvdH1100). Stably transfected cells were isolated and sub-cloned by limited dilution. Clones producing high levels of FSH were identified by ELISA (see below).

Example 3

[0232] Large-scale Production of FSH in CHO Cells

[0233] The cell line CHO K1 1100-5, stably expressing human FSH, was passed 1:10 from a confluent culture and propagated as adherent cells in serum-containing medium Dulbecco's MEM/Nut.-mix F-12 (Ham) L-glutamine, 15 mM Hepes, pyridoxine-HCl (Life Technologies Cat # 11039-021), 1:10 FBS (BioWhittaker Cat # 02-701F), 1:100 penicillin and streptomycin (BioWhittaker Cat # BE17-602E) until confluence in a 10 layer cell factory (NUNC #165250). The media was then changed to serum-free media: Dulbecco's MEM/Nut.-mix F-12 (Ham) L-glutamine, 15 mM Hepes, pyridoxine-HCl (Life Technologies Cat # 11039-021) with the addition of 1:500 ITS-A (Gibco/BRL # 51300-044), 1:500 EX-CYTE VLE (Serological Proteins Inc. # 81-129-1) and 1:100 penicillin and streptomycin (BioWhittaker Cat # BE17-602E). Subsequently, every 24 h, culture media were collected and replaced with 1 fresh liter of the same serum-free media. The collected media was filtered through 0.22 μm filters to remove cells. Growth in cell factories was continued with daily harvests and replacements of the culture media until FSH yields dropped below 25% of the initial expression level (typically after 10-15 days).

Example 4

[0234] Analysis of FSH Forms by Western Blotting and Isoelectric Focusing

[0235] The FSH content of samples was analysed by Western blotting: Proteins were separated by SDS-PAGE, and a standard Western blot was performed using rabbit anti human FSH (AHP519, Serotec) or mouse anti human FSH-β (MCA338, Serotec) as primary antibody, and an ImmunoPure Ultra Sensitive ABC Peroxidase Staining Kit (Pierce) for detection. Wild-type FSH produced as described above in Examples 1-3 was found to have the same mobility as FSH from references such as Puregon® (Organon) or Gonal-® (Serono).

[0236] For analysis of pI, samples were separated by on pH 3-7 IEF gels (NOVEX). After electrophoresis, proteins were blotted onto Immobilon-P (Millipore) membranes and a Western blot was performed as described above, using the same antibodies and detection kit. In accordance with published observations (see, for instance, Loumaye et al. (1998) *Human Reprod. Update* 4, 862-881), various FSH isoforms, mostly in the pH 4-5.2 range for wildtype FSH, were detected. This is due to heterogeneity in carbohydrate content, most importantly sialic acid.

Example 5

[0237] Purification of FSH Wildtype and Variants

[0238] Three chromatographic steps have been employed to obtain highly purified FSH. First an anion exchanger step, then hydrophobic interaction chromatography (HIC) and finally an immunoaffinity step using an FSH-β specific monoclonal antibody.

[0239] Culture supernatants were prepared as described in Example 3. Filtered culture supernatants were concentrated 10 to 20 times by ultrafiltration (10 kD cut-off membrane), pH was adjusted to 8.0 and conductivity to 10-15 mS/cm, before application on a DEAE Sepharose (Pharmacia) anion exchanger column, previously equilibrated in ammonium acetate buffer (0.16 M, pH 8.0). The binding capacity for a 25 ml (2.6×4.7 cm) column was sufficient to bind at least 0.5 mg FSH. Semipurified FSH was recovered both in the unbound flow-through fraction as well as in the wash fraction using 0.16 M ammonium acetate, pH 8.0. The flow through and wash fractions were pooled and ammonium sulfate was added from a stock solution (4.5 M) to obtain a final concentration of 1.5 M $(NH_4)_2SO_4$. The pH was adjusted to 7.0.

[0240] The partially purified FSH was subsequently applied on a 25 ml butyl Sepharose (Pharmacia) HIC column. After application, the column was washed with at least 3 column volumes of 1.5 M $(NH_4)_2SO_4$, 20 mM ammonium acetate, pH 7 (until the absorbance at 280 nm reached baseline level) and FSH was eluted with 4 column volumes of buffer B (20 mM ammonium acetate, pH 7). FSH enriched fractions from the HIC step were pooled, concentrated and diafiltrated using Vivaspin 20 modules, 10 kD cut-off membrane (Vivascience), to a 50 mM sodium phosphate, 150 mM NaCl, pH 7.2.

[0241] For the third chromatographic step, an anti-FSH-β monoclonal antibody (RDI-FSH909, Research Diagnostics) was immobilized to CNBr-activated Sepharose (Pharmacia) using a standard procedure from the supplier. Approximately 1 mg antibody was coupled per ml resin. The immunoaffinity resin was packed in plastic columns and equilibrated with 50 mM sodium phosphate, 150 mM NaCl, pH 7.2 before application.

[0242] The buffer exchanged eluate from the butyl HIC step was applied on the antibody column by use of gravity flow. This was followed by several washing steps in 50 mM sodium phosphate solutions (0.5 M NaCl and 1 M NaCl, both pH 7.2). Elution was performed using either 1 M NH_3 or 0.6 M NH_3, 40% (v/v) isopropanol and the eluate was immediately neutralized with 1 M acetic acid to pH 6-8.

[0243] The purified FSH bulk product was concentrated and diafiltrated using Vivaspin 20 modules, 10 kD cut-off membrane (Vivascience), to a 50 mM sodium phosphate, 150 mM NaCl, pH 7.2. For subsequent storage, BSA was added to 0.1% (w/v) and the purified FSH was microfiltrated using a 0.22 μm filter prior to storage at −80° C.

[0244] SDS-PAGE, run under non-dissociating conditions (without boiling), showed wildtype FSH migrating as an apparant 42±3 kDa band, slightly diffuse due to heterogeneity in the attached carbohydrates. The purity was about 80-90%. N-terminal sequencing showed that the α-chain had the expected N-terminal sequence starting with residue

1 (SEQ ID NO:2) and the β-chain starting with residue 3 (SEQ ID NO:4). These N-terminal sequences have been found previously for recombinant FSH produced in CHO cells (Olijve, W. et al. (1996) *Mol. Hum. Reprod.* 2, 371-382).

Example 6

[0245] FSH in Vitro Activity Assay

[0246] 6.1 FSH assay Outline

[0247] It has previously been published that activation of the FSH receptor by FSH leads to an increase in the intracellular concentration of cAMP. Consequently, transcription is activated at promoters containing multiple copies of the cAMP response element (CRE). It is thus possible to measure FSH activity by use of a CRE luciferase reporter gene introduced into CHO cells expressing the FSH receptor. 6.2 Construction of a CHO FSH-R/CRE-luc cell line

[0248] Stable clones expressing the human FSH receptor were produced by transfection of CHO K1 cells with a plasmid containing the receptor cDNA inserted into pcDNA3 (Invitrogen) followed by selection in media containing 600 μg/ml G418. Using a commercial cAMP-SPA RIA (Amersham), clones were screened for the ability to respond to FSH stimulation. On the basis of these results, an FSH receptor-expressing CHO clone was selected for further transfection with a CRE-luc reporter gene. A plasmid containing the reporter gene with 6 CRE elements in front of the Firefly luciferase gene was co-transfected with a plasmid conferring Hygromycin B resistance. Stable clones were selected in the presence of 600 μ/ml G418 and 400 μg/ml Hygromycin B. A clone yielding a robust luciferase signal upon stimulation with FSH (EC$_{50}$-0.01 IU/ml) was obtained. This CHO FSH-R/CRE-luc cell line was used to measure the activity of samples containing FSH. 6.3 FSH luciferase assay

[0249] To perform activity assays, CHO FSH-R/CRE-luc cells were seeded in white 96 well culture plates at a density of about 15,000 cells/well. The cells were in 100 μl DMEM/F-12 (without phenol red) with 1.25% FBS. After incubation overnight (at 37° C., 5% CO_2), 25 μl of sample or standard diluted in DMEM/F-12 (without phenol red) with 10% FBS was added to each well. The plates were further incubated for 3 hrs followed by addition of 125 μl LucLite substrate (Packard Bioscience). Subsequently, plates were sealed and luminescence was measured on a TopCount luminometer (Packard) in SPC (single photon counting) mode.

Example 7

[0250] FSH Elisa

[0251] The concentration of FSH in samples was quantified by use of a commercial immunoassay (DRG Instruments GmbH, Marburg, Germany). DRG FSH EIA is a solid phase immunosorbent assay (ELISA) based on the sandwich principle. The microtiter wells are coated with a monoclonal antibody directed towards a unique antigenic site on the FSH-β subunit. An aliquot of FSH-containing sample (diluted in H_2O with 0.1% BSA) and an anti-FSH antiserum conjugated with horseradish peroxidase are added to the coated wells. After incubation, unbound conjugate is washed off with water. The amount of bound peroxidase is proportional to the concentration of FSH in the sample. The intensity of colour developed upon addition of substrate solution is proportional to the concentration of FSH in the sample.

Example 8

[0252] Animal Studies

[0253] The pharmakinetic profile of FSH and variant forms was determined as follows: Immature 26-27 days old female Sprague-Dawley rats were injected i.v. with 3-4 μg FSH, and blood samples were taken at various time-points after injection. FSH concentrations in serum samples were determined by ELISA, as described in Example 7. In vivo bioactivity of wildtype recombinant FSH and variant forms can be evaluated by the ovarian weight augmentation assay (Steelman and Pohley (1953) *Endocrinology* 53, 604-616). Furthermore, the ability of FSH and variant forms to stimulate maturation of follicles in laboratory animals can be detected with e.g., ultrasound equipment.

Example 9

[0254] Construction and Abalysis of a Variant Form of FSH Containing Two N-linked Glycosylations at the N-terminus of the α Subunit

[0255] A construct encoding a modified form of FSH-α, having two additional sites for N-linked glycosylation at its N-terminus was generated by site-directed mutagenesis using standard DNA techniques known in the art. A DNA fragment encoding the sequence Ala-Asn-Ile-Thr-Val-Asn-He-Thr-Val was inserted immediately upstream of the mature FSH-α sequence in pBvdH977. The sequence of the resulting plasmid, termed pBvdH1163, is given in SEQ ID NO:7 (modified FSH-α-encoding sequence at position 1225 to 1599). A plasmid encoding both subunits was constructed by subcloning the FSH-containing NruI-PvuII fragment from pBvdH1163 into pBvdH1022 (Example 1), which had been linearized with PvuII. The resulting plasmid was termed pBvdH1208.

[0256] For expression of the variant form of FSH containing two N-linked glycosylations at the N-terminus of the α subunit (termed FSH1208), CHO K1 cells were transfected with pBvdH1208 or co-transfected with a combination of pBvdH1163, encoding the modified α subunit and pBvdH1022, encoding the wildtype β subunit. Transient expressions, isolation of stable expression clones, and large-scale production of FSH1208 were performed as described for wildtype FSH in Examples 2 and 3.

[0257] Western blotting showed that FSH1208 has a larger molecular mass than wildtype FSH, indicating that the introduction of acceptor sites for N-linked glycosylation at the N-terminus of the α subunit indeed leads to hyperglycosylation of FSH. Isoelectric focusing demonstrated that the FSH forms in the FSH1208 samples were found in a lower pI range than wildtype FSH produced as described in Examples 1-4. Thus, the pH interval for FSH1208 isoforms was about 3.0-4.5 versus about 4.0-5.2 for wildtype FSH. This indicated that FSH1208 molecules are on average more negatively charged than the wild type, which is attributed to the presence of additional sialic acid residues.

[0258] FSH1208 was purified and characterized as described in Examples 4 and 5. SDS-PAGE, run under non-dissociating conditions (without boiling), showed FSH1208 migrating as an apparent 55±5 kDa band, slightly diffuse due to heterogeneity in the attached carbohydrates. The purity was about 80-90%. N-terminal sequencing showed that while the β-chain had the same N-terminal

sequence as wildtype FSH, the sequence of α-chain was in agreement with this subunit carrying the expected N-terminal extension ANITVNITV, in which both asparagines residues are glycosylated.

[0259] The specific activity of FSH1208 was determined by measurement of the in vitro bioactivity (FSH luciferase assay, Example 6.3) and the FSH content of the samples (FSH ELISA, Examples). The specific activity of FSH1208 was found to be about one-third of that of the wildtype reference.

[0260] A pharmacokinetic study performed as described in Example 8 showed that 24 hours after injection of equal amounts of wildtype FSH and FSH1208, the sera of FSH1208-treated animals contained more than 10 fold more remaining immunoreactive material than the sera from animals treated with wildtype FSH.

Example 10

[0261] Construction and Analysis of other FSH Variants Containing Additional Glycosylation Sites

[0262] Plasmids encoding variant forms of FSH-α and FSH-β containing additional sites for N-linked glycosylation were generated by site-directed mutagenesis using standard DNA techniques known in the art. The following amino acid substitutions and/or insertions were generated:

[0263] FSH1147: Amino acid Tyr58 of mature FSH-β altered to Asn

[0264] FSH1349: N-terminus of mature FSH-α altered from APD QDC . . . to: APNDTVNFT QDC . . .

[0265] FSH1354: N-terminus of mature FSH-β altered from NS CEL . . . to: NSNITVNITV CEL . . .

[0266] Plasmids encoding the variant forms were transiently expressed in CHO K1 cells as described in Example 2. Plasmids encoding FSH-α variants were co-transfected with a plasmid encoding wild-type FSH-β and vice versa.

[0267] Western and isoelectric focusing were performed on culture media samples as described in Example 4. The variant forms had higher molecular weights than the wild-type, indicating that the additional acceptor sites for N-linked glycosylation had indeed been glycosylated. Furthermore, isoelectric focusing showed that the different isoforms of the three FSH variants were spread over a lower pI range than the wildtype. This strongly suggests that the variant forms had a higher sialic acid content than the wildtype.

[0268] In vitro FSH activities of the resulting media samples were analysed as described in Example 6.3. All three variant forms were able to stimulate the CHO FSH-R/CRE-luc cells, indicating that these variant FSH forms have retained significant FSH activity.

[0269] While the foregoing invention has been described in some detail for purposes of clarity and understanding, it will be clear to one skilled in the art from a reading of this disclosure that various changes in form and detail can be made without departing from the true scope of the invention. For example, all the techniques, methods, compositions, apparatus and systems described above can be used in various combinations. All publications, patents, patent applications, or other documents cited in this application are incorporated by reference in their entirety for all purposes to the same extent as if each individual publication, patent, patent

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        35                  40                  45

Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser
    50                  55                  60

Tyr Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr
65                  70                  75                  80

Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
                85                  90


<210> SEQ ID NO 3
<211> LENGTH: 129
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 3

Met Lys Thr Leu Gln Phe Phe Phe Leu Phe Cys Cys Trp Lys Ala Ile
 1               5                   10                  15

Cys Cys Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys
            20                  25                  30

Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly
        35                  40                  45

Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys
    50                  55                  60

Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg
65                  70                  75                  80

Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val
                85                  90                  95

Ala Thr Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys
            100                 105                 110

Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu Met Lys
        115                 120                 125

Glu


<210> SEQ ID NO 4
<211> LENGTH: 111
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 4

Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys Glu Glu
 1               5                   10                  15
```

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```
Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly Tyr Cys
            20                  25                  30

Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys Ile Gln
            35                  40                  45

Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg Val Pro
    50                  55                  60

Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val Ala Thr
65                  70                  75  .               80

Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys Thr Val
                85                  90                  95

Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu Met Lys Glu
            100                 105                 110
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<210> SEQ ID NO 5
<211> LENGTH: 6186
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1225)..(1572)

<400> SEQUENCE: 5
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ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg     120

cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc     180

ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt     240

gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata     300

tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc     360

cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc     420

attgacgtca atgggtggac tatttacggt aaactgccca cttggcagta catcaagtgt     480

atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt     540

atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca     600

tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg     660

actcacgggg atttccaagt ctccacccca ttgacgtcaa tgggagtttg ttttggcacc     720

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ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagc     900

ttattgcggt agtttatcac agttaaattg ctaacgcagt cagtgcttct gacacaacag     960

tctcgaactt aagctgcagt gactctctta aggtagcctt gcagaagttg gtcgtgaggc    1020

actgggcagg taagtatcaa ggttacaaga caggtttaag agaccaata gaaactgggc    1080

ttgtcgagac agagaagact cttgcgtttc tgataggcac ctattggtct tactgacatc    1140

cactttgcct ttctctccac aggtgtccac tcccagttca attacagctc ttaaaagctt    1200
```

```
ggtaccgagc tcggatccgc cacc atg gac tac tac cgc aag tac gcc gcc      1251
                              Met Asp Tyr Tyr Arg Lys Tyr Ala Ala
                              1               5
```

```
atc ttc ctg gtg acc ctg agc gtg ttc ctg cac gtg ctg cac agc gcc      1299
Ile Phe Leu Val Thr Leu Ser Val Phe Leu His Val Leu His Ser Ala
10                  15                  20                  25
```

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ccc gac gtg cag gac tgc ccc gag tgc acc ctg cag gag aac ccc ttc      1347
Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro Phe
            30                  35                  40

ttc agc cag ccc ggc gcc ccc atc ctg cag tgc atg ggc tgc tgc ttc      1395
Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe
            45                  50                  55

agc cgc gcc tac ccc acc ccc ctg cgc agc aag aag acc atg ctg gtg      1443
Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val
        60                  65                  70

cag aag aac gtg acc agc gag agc acc tgc tgc gtg gcc aag agc tac      1491
Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr
    75                  80                  85

aac cgc gtg acc gtg atg ggc ggc ttc aag gtg gag aac cac acc gcc      1539
Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala
90                  95                  100                 105

tgc cac tgc agc acc tgc tac tac cac aag agc taatctagag ggcccgttta    1592
Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
                110                 115

aacccgctga tcagcctcga ctgtgccttc tagttgccag ccatctgttg tttgcccctc     1652

ccccgtgcct tccttgaccc tggaaggtgc cactcccact gtcctttcct aataaaatga     1712

ggaaattgca tcgcattgtc tgagtaggtg tcattctatt ctgggggggtg gggtggggca    1772

ggacagcaag ggggaggatt gggaagacaa tagcaggcat gctgggggatg cggtgggctc    1832

tatggcttct gaggcggaaa gaaccagctg gggctctagg gggtatcccc acgcgccctg     1892

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cagcgcccta gcgcccgctc ctttcgcttt cttcccttcc tttctcgcca cgttcgccgg     2012

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gcacctcgac cccaaaaaac ttgattaggg tgatggttca cgtagtgggc catcgccctg     2132

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ccaaactgga acaacactca accctatctc ggtctattct tttgatttat aagggatttt     2252

ggggatttcg gcctattggt taaaaaatga gctgatttaa caaaaattta acgcgaatta     2312

attctgtgga atgtgtgtca gttagggtgt ggaaagtccc caggctcccc aggcaggcag     2372

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cctaactccg cccatcccgc ccctaactcc gcccagttcc gcccattctc cgccccatgg     2552

ctgactaatt ttttttattt atgcagaggc cgaggccgcc tctgcctctg agctattcca     2612

gaagtagtga ggaggctttt ttggaggcct aggcttttgc aaaaagctcc cgggagcttg     2672

tatatccatt ttcggatctg atcagcacgt gatgaaaaag cctgaactca ccgcgacgtc     2732

tgtcgagaag tttctgatcg aaaagttcga gcgtctcc gacctgatgc agctctcgga      2792

gggcgaagaa tctcgtgctt tcagcttcga tgtaggaggg cgtggatatg tcctgcgggt     2852

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catctcccgc cgtgcacagg gtgtcacgtt gcaagacctg cctgaaaccg aactgcccgc     3032

tgttctgcag ccggtcgcgg aggccatgga tgcgatcgct gcggccgatc ttagccagac     3092

gagcgggttc ggcccattcg gaccgcaagg aatcggtcaa tacactacat ggcgtgattt     3152
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catatgcgcg attgctgatc cccatgtgta tcactggcaa actgtgatgg acgacaccgt    3212

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cataacagcg gtcattgact ggagcgaggc gatgttcggg gattcccaat acgaggtcgc    3392

caacatcttc ttctggaggc cgtggttggc ttgtatggag cagcagacgc gctacttcga    3452

gcggaggcat ccggagcttg caggatcgcc gcggctccgg gcgtatatgc tccgcattgg    3512

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agcttataat ggttacaaat aaagcaatag catcacaaat ttcacaaata aagcattttt    3932

ttcactgcat tctagttgtg gtttgtccaa actcatcaat gtatcttatc atgtctgtat    3992

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ttgttatccg ctcacaattc cacacaacat acgagccgga agcataaagt gtaaagcctg    4112

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tttgcgtatt gggcgctctt ccgcttcctc gctcactgac tcgctgcgct cggtcgttcg    4292

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ttaaaaatga agttttaaat caatctaaag tatatatgag taaacttggt ctgacagtta    5192

ccaatgctta atcagtgagg cacctatctc agcgatctgt ctatttcgtt catccatagt    5252

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tagctccttc ggtcctccga tcgttgtcag aagtaagttg gccgcagtgt tatcactcat    5672

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gactggtgag tactcaacca agtcattctg agaatagtgt atgcggcgac cgagttgctc    5792

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cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc ttaccgctgt tgagatccag    5912

ttcgatgtaa cccactcgtg cacccaactg atcttcagca tctttactt tcaccagcgt     5972

ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa aagggaataa gggcgacacg    6032

gaaatgttga atactcatac tcttcctttt tcaatattat tgaagcattt atcagggtta    6092

ttgtctcatg agcggataca tatttgaatg tatttagaaa ataaacaaa tagggggttcc    6152

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<210> SEQ ID NO 6
<211> LENGTH: 5651
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1231)..(1617)

<400> SEQUENCE: 6

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cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc    180

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ttgtcgagac agagaagact cttgcgtttc tgataggcac ctattggtct tactgacatc    1140
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                                  Met Glu Thr Leu Gln Phe Phe Phe
                                   1               5

ctg ttc tgc tgc tgg aag gcc atc tgc tgc aac agc tgc gag ctg acc        1302
Leu Phe Cys Cys Trp Lys Ala Ile Cys Cys Asn Ser Cys Glu Leu Thr
        10                  15                  20

aac atc acc atc gcc atc gag aag gag gag tgc cgc ttc tgc atc agc        1350
Asn Ile Thr Ile Ala Ile Glu Lys Glu Glu Cys Arg Phe Cys Ile Ser
 25                  30                  35                  40

atc aac acc acc tgg tgc gcc ggc tac tgc tac acc cgc gac ctg gtg        1398
Ile Asn Thr Thr Trp Cys Ala Gly Tyr Cys Tyr Thr Arg Asp Leu Val
                45                  50                  55

tac aag gac ccc gcc cgc ccc aag atc cag aag acc tgc acc ttc aag        1446
Tyr Lys Asp Pro Ala Arg Pro Lys Ile Gln Lys Thr Cys Thr Phe Lys
                    60                  65                  70

gag ctg gtg tac gag acg gtc cgg gtg ccc ggc tgc gcc cac cac gcc        1494
Glu Leu Val Tyr Glu Thr Val Arg Val Pro Gly Cys Ala His His Ala
            75                  80                  85

gac agc ctg tac acc tac ccc gtg gcc acc cag tgc cac tgc ggc aag        1542
Asp Ser Leu Tyr Thr Tyr Pro Val Ala Thr Gln Cys His Cys Gly Lys
        90                  95                  100

tgc gac agc gac agc acc gac tgc acc gtg cgc ggc ctg ggc ccc agc        1590
Cys Asp Ser Asp Ser Thr Asp Cys Thr Val Arg Gly Leu Gly Pro Ser
105                 110                 115                 120

tac tgc agc ttc ggc gag atg aag gag taactcgaga ctagagggcc              1637
Tyr Cys Ser Phe Gly Glu Met Lys Glu
                125

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cttgttccaa actggaacaa cactcaaccc tatctcggtc tattcttttg atttataagg        2297

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attccagaag tagtgaggag gcttttttgg aggcctaggc ttttgcaaaa agctcccggg        2717

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agcataaagt gtaaagcctg gggtgcctaa tgagtgagct aactcacatt aattgcgttg    3617

cgctcactgc ccgctttcca gtcgggaaac ctgtcgtgcc agctgcatta atgaatcggc    3677

caacgcgcgg ggagaggcgg tttgcgtatt gggcgctctt ccgcttcctc gctcactgac    3737

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gatttatcag caataaacca gccagccgga agggccgagc gcagaagtgg tcctgcaact    4877

ttatccgcct ccatccagtc tattaattgt tgccgggaag ctagagtaag tagttcgcca    4937

gttaatagtt tgcgcaacgt tgttgccatt gctacaggca tcgtggtgtc acgctcgtcg    4997

tttggtatgg cttcattcag ctccggttcc caacgatcaa ggcgagttac atgatccccc    5057
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atgttgtgca aaaaagcggt tagctccttc ggtcctccga tcgttgtcag aagtaagttg      5117

gccgcagtgt tatcactcat ggttatggca gcactgcata attctcttac tgtcatgcca      5177

tccgtaagat gcttttctgt gactggtgag tactcaacca agtcattctg agaatagtgt      5237

atgcggcgac cgagttgctc ttgcccggcg tcaatacggg ataataccgc gccacatagc      5297

agaactttaa aagtgctcat cattggaaaa cgttcttcgg ggcgaaaact ctcaaggatc      5357

ttaccgctgt tgagatccag ttcgatgtaa cccactcgtg cacccaactg atcttcagca      5417

tcttttactt tcaccagcgt ttctgggtga gcaaaaacag gaaggcaaaa tgccgcaaaa      5477

aagggaataa gggcgacacg gaaatgttga atactcatac tcttcctttt tcaatattat      5537

tgaagcattt atcagggtta ttgtctcatg agcggataca tatttgaatg tatttagaaa      5597

aataaacaaa taggggttcc gcgcacattt ccccgaaaag tgccacctga cgtc           5651
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<210> SEQ ID NO 7
<211> LENGTH: 6213
<212> TYPE: DNA
<213> ORGANISM: Homo sapiens
<220> FEATURE:
<221> NAME/KEY: CDS
<222> LOCATION: (1225)..(1599)

<400> SEQUENCE: 7
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ccgcatagtt aagccagtat ctgctccctg cttgtgtgtt ggaggtcgct gagtagtgcg      120

cgagcaaaat ttaagctaca acaaggcaag gcttgaccga caattgcatg aagaatctgc      180

ttagggttag gcgttttgcg ctgcttcgcg atgtacgggc cagatatacg cgttgacatt      240

gattattgac tagttattaa tagtaatcaa ttacggggtc attagttcat agcccatata      300

tggagttccg cgttacataa cttacggtaa atggcccgcc tggctgaccg cccaacgacc      360

cccgcccatt gacgtcaata atgacgtatg ttcccatagt aacgccaata gggactttcc      420

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atcatatgcc aagtacgccc cctattgacg tcaatgacgg taaatggccc gcctggcatt      540

atgcccagta catgacctta tgggactttc ctacttggca gtacatctac gtattagtca      600

tcgctattac catggtgatg cggttttggc agtacatcaa tgggcgtgga tagcggtttg      660

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ctgcttactg gcttatcgaa attaatacga ctcactatag ggagacccaa gctggctagc      900

ttattgcggt agtttatcac agttaaattg ctaacgcagt cagtgcttct gacacaacag      960

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actgggcagg taagtatcaa ggttacaaga caggtttaag gagaccaata gaaactgggc     1080

ttgtcgagac agagaagact cttgcgtttc tgataggcac ctattggtct tactgacatc     1140

cactttgcct ttctctccac aggtgtccac tcccagttca attacagctc ttaaaagctt     1200

ggtaccgagc tcggatccgc cacc atg gac tac tac cgc aag tac gcc gcc        1251
                              Met Asp Tyr Tyr Arg Lys Tyr Ala Ala
                               1               5

atc ttc ctg gtg acc ctg agc gtg ttc ctg cac gtg ctg cac agc gcc       1299
```

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```
Ile Phe Leu Val Thr Leu Ser Val Phe Leu His Val Leu His Ser Ala
10              15              20              25

aac atc acc gtt aac atc acc gtg gcc ccc gac gtg cag gac tgc ccc   1347
Asn Ile Thr Val Asn Ile Thr Val Ala Pro Asp Val Gln Asp Cys Pro
                30              35              40

gag tgc acc ctg cag gag aac ccc ttc ttc agc cag ccc ggc gcc ccc   1395
Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln Pro Gly Ala Pro
            45              50              55

atc ctg cag tgc atg ggc tgc tgc ttc agc cgc gcc tac ccc acc ccc   1443
Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala Tyr Pro Thr Pro
        60              65              70

ctg cgc agc aag aag acc atg ctg gtg cag aag aac gtg acc agc gag   1491
Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn Val Thr Ser Glu
    75              80              85

agc acc tgc tgc gtg gcc aag agc tac aac cgc gtg acc gtg atg ggc   1539
Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val Thr Val Met Gly
90              95              100             105

ggc ttc aag gtg gag aac cac acc gcc tgc cac tgc agc acc tgc tac   1587
Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys Ser Thr Cys Tyr
            110             115             120

tac cac aag agc taatctagag ggcccgttta aacccgctga tcagcctcga       1639
Tyr His Lys Ser
            125

ctgtgccttc tagttgccag ccatctgttg tttgcccctc ccccgtgcct tccttgaccc   1699

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tgagtaggtg tcattctatt ctggggggtg gggtggggca ggacagcaag ggggaggatt   1819

gggaagacaa tagcaggcat gctggggatg cggtgggctc tatggcttct gaggcggaaa   1879

gaaccagctg gggctctagg gggtatcccc acgcgccctg tagcggcgca ttaagcgcgg   1939

cgggtgtggt ggttacgcgc agcgtgaccg ctacacttgc cagcgcccta gcgcccgctc   1999

ctttcgcttt cttcccttcc tttctcgcca cgttcgccgg ctttccccgt caagctctaa   2059

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tgacgttgga gtccacgttc tttaatagtg gactcttgtt ccaaactgga acaacactca   2239

accctatctc ggtctattct tttgatttat aagggatttt ggggatttcg gcctattggt   2299

taaaaaatga gctgatttaa caaaaattta acgcgaatta attctgtgga atgtgtgtca   2359

gttagggtgt ggaaagtccc caggctcccc aggcaggcag aagtatgcaa agcatgcatc   2419

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ccctaactcc gcccagttcc gcccattctc cgccccatgg ctgactaatt tttttattt   2599

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tctacaaaga tcgttatgtt tatcggcact ttgcatcggc cgcgctcccg attccggaag   2959

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gtgtcacgtt gcaagacctg cctgaaaccg aactgcccgc tgttctgcag ccggtcgcgg    3079

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gaccgcaagg aatcggtcaa tacactacat ggcgtgattt catatgcgcg attgctgatc    3199

cccatgtgta tcactggcaa actgtgatgg acgacaccgt cagtgcgtcc gtcgcgcagg    3259

ctctcgatga gctgatgctt tgggccgagg actgccccga agtccggcac ctcgtgcacg    3319

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caggatcgcc gcggctccgg gcgtatatgc tccgcattgg tcttgaccaa ctctatcaga    3559

gcttggttga cggcaatttc gatgatgcag cttgggcgca gggtcgatgc gacgcaatcg    3619

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ccgcttcctc gctcactgac tcgctgcgct cggtcgttcg gctgcggcga gcggtatcag    4339

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caatctaaag tatatatgag taaacttggt ctgacagtta ccaatgctta atcagtgagg    5239

cacctatctc agcgatctgt ctatttcgtt catccatagt tgcctgactc cccgtcgtgt    5299
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agtcattctg agaatagtgt atgcggcgac cgagttgctc ttgcccggcg tcaatacggg    5839

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cacccaactg atcttcagca tcttttactt tcaccagcgt ttctgggtga gcaaaaacag    6019

gaaggcaaaa tgccgcaaaa aagggaataa gggcgacacg gaaatgttga atactcatac    6079

tcttcctttt tcaatattat tgaagcattt atcagggtta ttgtctcatg agcggataca    6139

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<210> SEQ ID NO 8
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 8

Asn Ser Thr Gln Asn Ala Thr Ala
 1               5


<210> SEQ ID NO 9
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 9

Ala Asn Leu Thr Val Arg Asn Leu Thr Arg Asn Val Thr Val
 1               5                   10


<210> SEQ ID NO 10
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 10

Ala Asn Ile Thr Val Asn Ile Thr Val
 1               5
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<210> SEQ ID NO 11
<211> LENGTH: 7
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 11

Asn Asp Thr Val Asn Phe Thr
  1               5


<210> SEQ ID NO 12
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 12

Asn Ile Thr Val Asn Ile Thr Val
  1               5


<210> SEQ ID NO 13
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 13

Ala Ala Thr Pro Ala Pro
  1               5


<210> SEQ ID NO 14
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 14

His His His His His His
  1               5


<210> SEQ ID NO 15
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 15

Met Lys His His His His His His
  1               5


<210> SEQ ID NO 16
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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      peptide

<400> SEQUENCE: 16

Met Lys His His Ala His His Gln His His
 1               5                  10


<210> SEQ ID NO 17
<211> LENGTH: 14
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 17

Met Lys His Gln His Gln His Gln His Gln His Gln His Gln
 1               5                  10


<210> SEQ ID NO 18
<211> LENGTH: 15
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 18

Met Lys His Gln His Gln His Gln His Gln His Gln His Gln Gln
 1               5                  10                 15


<210> SEQ ID NO 19
<211> LENGTH: 10
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      peptide

<400> SEQUENCE: 19

Glu Gln Lys Leu Ile Ser Glu Glu Asp Leu
 1               5                  10


<210> SEQ ID NO 20
<211> LENGTH: 8
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      peptide

<400> SEQUENCE: 20

Asp Tyr Lys Asp Asp Asp Asp Lys
 1               5


<210> SEQ ID NO 21
<211> LENGTH: 9
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 21

Tyr Pro Tyr Asp Val Pro Asp Tyr Ala
 1               5
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<210> SEQ ID NO 22
<211> LENGTH: 5
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
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<400> SEQUENCE: 22

Gly Gly Gly Gly Ser
  1               5


<210> SEQ ID NO 23
<211> LENGTH: 129
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 23

Met Glu Thr Leu Gln Phe Phe Phe Leu Phe Cys Cys Trp Lys Ala Ile
  1               5                  10                  15

Cys Cys Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala Ile Glu Lys
              20                  25                  30

Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp Cys Ala Gly
          35                  40                  45

Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala Arg Pro Lys
      50                  55                  60

Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu Thr Val Arg
 65                  70                  75                  80

Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr Tyr Pro Val
                  85                  90                  95

Ala Thr Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser Thr Asp Cys
              100                 105                 110

Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly Glu Met Lys
          115                 120                 125

Glu


<210> SEQ ID NO 24
<211> LENGTH: 125
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 24

Met Asp Tyr Tyr Arg Lys Tyr Ala Ala Ile Phe Leu Val Thr Leu Ser
  1               5                  10                  15

Val Phe Leu His Val Leu His Ser Ala Asn Ile Thr Val Asn Ile Thr
              20                  25                  30

Val Ala Pro Asp Val Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn
          35                  40                  45

Pro Phe Phe Ser Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys
      50                  55                  60

Cys Phe Ser Arg Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met
 65                  70                  75                  80

Leu Val Gln Lys Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys
                  85                  90                  95

Ser Tyr Asn Arg Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His
```

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```
            100                 105                 110

Thr Ala Cys His Cys Ser Thr Cys Tyr Tyr His Lys Ser
        115                 120                 125


<210> SEQ ID NO 25
<211> LENGTH: 6
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 25

Ala Pro Asp Gln Asp Cys
 1               5


<210> SEQ ID NO 26
<211> LENGTH: 12
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      peptide

<400> SEQUENCE: 26

Ala Pro Asn Asp Thr Val Asn Phe Thr Gln Asp Cys
 1               5                   10


<210> SEQ ID NO 27
<211> LENGTH: 13
<212> TYPE: PRT
<213> ORGANISM: Artificial Sequence
<220> FEATURE:
<223> OTHER INFORMATION: Description of Artificial Sequence: Synthetic
      peptide

<400> SEQUENCE: 27

Asn Ser Asn Ile Thr Val Asn Ile Thr Val Cys Glu Leu
 1               5                   10


<210> SEQ ID NO 28
<211> LENGTH: 196
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 28

Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln
 1               5                   10                  15

Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala
            20                  25                  30

Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn
        35                  40                  45

Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val
    50                  55                  60

Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys
65                  70                  75                  80

Ser Thr Cys Tyr Tyr Asn Ser Cys Glu Leu Thr Asn Ile Thr Ile Ala
                85                  90                  95

Ile Glu Lys Glu Glu Cys Arg Phe Cys Ile Ser Ile Asn Thr Thr Trp
            100                 105                 110

Cys Ala Gly Tyr Cys Tyr Thr Arg Asp Leu Val Tyr Lys Asp Pro Ala
        115                 120                 125
```

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Arg Pro Lys Ile Gln Lys Thr Cys Thr Phe Lys Glu Leu Val Tyr Glu
    130   135   140

Thr Val Arg Val Pro Gly Cys Ala His His Ala Asp Ser Leu Tyr Thr
145   150   155   160

Tyr Pro Val Ala Thr Gln Cys His Cys Gly Lys Cys Asp Ser Asp Ser
                165   170   175

Thr Asp Cys Thr Val Arg Gly Leu Gly Pro Ser Tyr Cys Ser Phe Gly
            180   185   190

Glu Met Lys Glu
        195

<210> SEQ ID NO 29
<211> LENGTH: 196
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 29

Thr Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser
  1   5   10   15

Gln Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg
            20   25   30

Ala Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys
        35   40   45

Asn Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg
    50   55   60

Val Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His
65   70   75   80

Cys Ser Thr Cys Tyr Tyr Lys Glu Pro Leu Arg Pro Arg Cys Arg Pro
                85   90   95

Ile Asn Ala Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile
            100   105   110

Thr Val Asn Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg
        115   120   125

Val Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr
    130   135   140

Arg Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly
145   150   155   160

Val Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala
                165   170   175

Leu Cys Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro
            180   185   190

Leu Thr Cys Asp
        195

<210> SEQ ID NO 30
<211> LENGTH: 195
<212> TYPE: PRT
<213> ORGANISM: Homo sapiens

<400> SEQUENCE: 30

Gln Asp Cys Pro Glu Cys Thr Leu Gln Glu Asn Pro Phe Phe Ser Gln
  1   5   10   15

Pro Gly Ala Pro Ile Leu Gln Cys Met Gly Cys Cys Phe Ser Arg Ala
            20   25   30

-continued

```
Tyr Pro Thr Pro Leu Arg Ser Lys Lys Thr Met Leu Val Gln Lys Asn
        35              40              45

Val Thr Ser Glu Ser Thr Cys Cys Val Ala Lys Ser Tyr Asn Arg Val
    50              55              60

Thr Val Met Gly Gly Phe Lys Val Glu Asn His Thr Ala Cys His Cys
65              70              75              80

Ser Thr Cys Tyr Tyr Lys Glu Pro Leu Arg Pro Arg Cys Arg Pro Ile
            85              90              95

Asn Ala Thr Leu Ala Val Glu Lys Glu Gly Cys Pro Val Cys Ile Thr
        100             105             110

Val Asn Thr Thr Ile Cys Ala Gly Tyr Cys Pro Thr Met Thr Arg Val
        115             120             125

Leu Gln Gly Val Leu Pro Ala Leu Pro Gln Val Val Cys Asn Tyr Arg
    130             135             140

Asp Val Arg Phe Glu Ser Ile Arg Leu Pro Gly Cys Pro Arg Gly Val
145             150             155             160

Asn Pro Val Val Ser Tyr Ala Val Ala Leu Ser Cys Gln Cys Ala Leu
            165             170             175

Cys Arg Arg Ser Thr Thr Asp Cys Gly Gly Pro Lys Asp His Pro Leu
            180             185             190

Thr Cys Asp
        195
```

What is claimed is:

1. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising

   i) a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein at least one of said FSH-α and FSH-β subunits differs from the corresponding wildtype subunit in that at least one amino acid residue acid residue comprising an attachment group for a non-polypeptide moiety has been introduced or removed, and

   ii) at least one non-polypeptide moiety bound to an attachment group of at least one of said subunits.

2. The conjugate of claim 1, wherein the amino acid sequence of at least one of said FSH-α and FSH-β subunits differs from that of the corresponding wildtype subunit in that an amino acid residue comprising an attachment group for the non-polypeptide moiety has been introduced.

3. The conjugate of claim 2, wherein the introduced attachment group is selected from the group consisting of an N-glycosylation site, an O-glycosylation site, and an attachment group for a polymer molecule, a lipophilic compound, a carbohydrate moiety or an organic derivatizing agent.

4. The conjugate of claim 1, comprising at least one PEG molecule attached to an attachment group of at least one of the subunits.

5. The conjugate of claim 1, comprising at least one introduced N-glycosylation site, and further comprising at least one PEG molecule attached to an attachment group of at least one of the subunits.

6. The conjugate of claim 5, wherein said at least one PEG molecule is bound to the N-terminal of at least one of the subunits.

7. The conjugate of claim 1, wherein the amino acid sequence of the FSH-α subunit differs from that of wildtype human FSH-α.

8. The conjugate of claim 1, wherein the amino acid sequence of the FSH-β subunit differs from that of wildtype human FSH-β.

9. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising

   i) a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein the amino acid sequence of at least one of said FSH-α and FSH-β subunits differs from that of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced, and

   ii) at least one oligosaccharide moiety bound to an N-glycosylation site of at least one of said subunits.

10. The conjugate of claim 9, wherein at least one N-glycosylation site has been introduced into the FSH-α subunit by a mutation selected from the group consisting of P2(a)N+V4(a)S, P2(a)N+V4(a)T, D3(a)N+Q5(a)S, D3(a)N+Q5(a)T, V4(a)N+D6(a)S, V4(a)N+D6(a)S, D6(a)N+P8(a)S, D6(a)N+P8(a)T, E9(a)N+T11(a)S, E9(a)N, T11(a)N+Q13(a)S, T11(a)N+Q13(a)T, L12(a)N+E14(a)S, L12(a)N+E14(a)T, E14(a)N+P16(a)S, E14(a)N+P16(a)T, P16(a)N+F18(a)S, P16(a)N+F18(a)T, F17(a)N, F17(a)N+ S19(a)T, G22(a)N+P24(a)S, G22(a)N+P24(a)T, P24(a)N+ L26(a)S, P24(a)N+L26(a)T, F33(a)N+R35(a)S, F33(a)N+ R35(a)T, R42(a)N+K44(a)S, R42(a)N+K44(a)T, S43(a)N+ K45(a)S, S43(a)N+K45(a)T, K44(a)N+T46(a)S, K44(a)N, K45(a)N+M47(a)S, K45(a)N+M47(a)T, T46(a)N+L48(a)S, T46(a)N+L48(a)T, L48(a)N+Q50(a)S, 148(a)N+Q50(a)T, V49(a)N+H51(a)S, V49(a)N+K51(a)T, Q50(a)N+N52(a)S,

Q50(a)N+N52(a)T, V61(a)N+K63(a)S, V61(a)N+K63(a)T, K63(a)N+Y65(a)S, K63(a)N+Y65(a)T, S64(a)N+N66(a)S, S64(a)N+N66(a)T, Y65(a)N+R67(a)S, Y65(a)N+R67(a)T, V68(a)S, V68(a)T, R67(a)N+T69(a)S, R67(a)N, T69(a)N+ M71(a)S, T69(a)N+M71(a)T, M71(a)N+G73(a)S, M71(a)N+G73(a)T, G72(a)N+F74(a)S, G72(a)N+F74(a)T, G73(a)N+K75(a)S, G73(a)N+K75(a)T, F74(a)N+V76(a)S, F74(a)N+V76(a)T, K75(a)N+E77(a)S, K75(a)N+E77(a)T, A81(a)N+I183(a)S, A81(a)N+H83(a)T, H83(a)N, T86(a)N+ Y88(a)S, T86(a)N+Y88(a)T, Y88(a)N+I190(a)S, Y88(a)N+ H90(a)T, Y89(a)N+K91(a)S, Y89(a)N+K91(a)T, H90(a)N and H90(a)N+S92(a)T.

11. The conjugate of claim 9, wherein at least one N-glycosylation site has been introduced into the FSH-β subunit by a mutation selected from the group consisting of S2(b)N+E4(b)S, S2(b)N+E4(b)T, E4(b)N+T6(b)S, E4(b)N, L5(b)N+N7(b)S, L5(b)N+L7(b)T, T6(b)N+I8(b)S, T6(b)N+ I8(b)T, I8(b)N+I10(b)S, I8(b)N+I10(b)T, T9(b)N+A11(b)S, T9(b)N+A11(b)T, K14(b)N+E16(b)S, K14(b)N+E16(b)T, F19(b)N+I21(b)S, F19(b)N+I21(b)T, I21(b)N+I23(b)S, I21(b)N+I23(b)T, S22(b)N+N24(b)S, S22(b)N+N24(b)T, Y31(b)N+Y33(b)S, Y31(b)N+Y33(b)T, Y33(b)N+R35(b)S, Y33(b)N+R35(b)T, R35(b)N+L37(b)S, R35(b)N+L37(b)T, D36(b)N+V38(b)S, D36(b)N+V38(b)T, L37(b)N+Y39(b)S, L37(b)N+Y39(b)T, K40(b)N+P42(b)S, K40(b)N+P42(b)T, A43(b)N+P45(b)S, A43(b)N+P45(b)T, P45(b)N+I47(b)S, P45(b)N+I47(b)T, K46(b)N+Q48(b)S, K46(b)N+Q48(b)T, I47(b)N+K49(b)S, I47(b)N+K49(b)T, K54(b)N+L56(b)S, K54(b)N+L56(b)T, E55(b)N+V57(b)S, E55(b)N+V57(b)T, L56(b)N+Y58(b)S, L56(b)N+Y58(b)T, V57(b)N+E59(b)S, V57(b)N+E59(b)T, Y58(b)N+T60(b)S, Y58(b)N, E59(b)N+ V61(b)S, E59(b)N+V61(b)T, T60(b)N+R62(b)S, T60(b)N+ R62(b)T, R62(b)N+P64(b)S, R62(b)N+P64(b)T, G65(b)N+ A67(b)S, G65(b)N+A67(b)T, A67(b)N+H69(b)S, A67(b)N+H69(b)T, H68(b)N+A70(b)S, H68(b)N+A70(b)T, H69(b)N+D71(b)S, H69(b)N+D71(b)T, D71(b)N+L73(b)S, D71(b)N+L73(b)T, L73(b)N+T75(b)S, L73(b)N, T75(b)N+ P77(b)S, T75(b)N+P77(b)T, H83(b)N+G85(b)S, H83(b)N+ G85(b)T, K86(b)N+D88(b)S, K86(b)N+D88(b)T, D88(b)N+D90(b)S, D88(b)N+D90(b)T, S89(b)N, S89(b)N+ S91(b)T, D90(b)N+T92(b)S, D90(b)N, S91(b)N+D93(b)S, S91(b)N+D93(b)T, D93(b)N+T96(b)S, D93(b)N, T95(b)N+ R97(b)S, T95(b)N+R97(b)T, V96(b)N+G98(b)S, V96(b)N+ G98(b)T, R97(b)N+L99(b)S, R97(b)N+L99(b)T, L99(b)N+ P101(b)S, L99(b)N+P101(b)T, Y103(b)N, Y103(b)N+ S105(b)T, S105(b)N+G107(b)S, S105(b)N+G107(b)T, F106(b)N+E108(b)S, F106(b)N+E108(b)T, G107(b)N+ M109(b)S, G107(b)N+M109(b)T, E108(b)N+K110(b)S, E108(b)N+K110(b)T, M109(b)N+E111(b)S, and M109(b)N+E111(b)T.

12. The conjugate of claim 9, wherein at least one of the FSH-α and FSH-β subunits comprises at least one N- or C-terminal peptide addition comprising at least one N-glycosylation site.

13. The conjugate of claim 9, which further comprises at least one non-polypeptide moiety different from an N- or O-linked oligosaccharide moiety bound to an attachment group of the polypeptide.

14. The conjugate of claim 9, wherein the amino acid sequence of at least one of said FSH-α and FSH-β subunits further differs from that of the corresponding wildtype subunit in that at least one naturally occurring N-glycosylation site has been removed.

15. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a dimeric polypeptide comprising an FSH-α subunit and an FSH-β subunit, wherein at least one of said FSH-α and FSH-β subunits comprises a polymer molecule bound to the N-terminal thereof.

16. The conjugate of claim 15, wherein the polymer molecule is polyethylene glycol.

17. The conjugate of claim 15, wherein at least one of said FSH-α and FSH-β subunit comprises, relative to the corresponding wildtype human subunit, at least one introduced amino acid residue comprising an attachment group for the polymer molecule, and/or wherein at least one amino acid residue comprising an attachment group for a polymer molecule has been removed.

18. A heterodimeric polypeptide conjugate exhibiting FSH activity, comprising a dimeric polypeptide comprising FSH-α and FSH-β subunits, wherein at least one of said FSH-α and FSH-β subunits comprises, relative to the corresponding wildtype subunit, at least one introduced N- or O-glycosylation site at the N-terminal thereof, said at least one introduced glycosylation site being glycosylated.

19. The conjugate of claim 18, wherein said at least one introduced N- or O-glycosylation site is part of an N-terminal peptide addition.

20. The conjugate of claim 1, wherein the FSH-α subunit comprises hFSH-α having the sequence shown in SEQ ID NO:2, or the FSH-β subunit comprises hFSH-β having the sequence shown in SEQ ID NO:4.

21. The conjugate of claim 1, wherein the amino acid sequence of the FSH-α and/or FSH-β subunit differs in 1-20 amino acid residues from that of the corresponding wildtype sequence.

22. The conjugate of claim 1, which has an increased functional in vivo half-life and/or serum half-life as compared to hFSH.

23. The conjugate of claim 1, wherein the FSH-α subunit and the FSH-β subunit are linked by a peptide bond or a peptide linker to form a single-chain polypeptide.

24. A composition comprising a conjugate according to claim 1 and at least one pharmaceutically acceptable carrier or excipient.

25. A composition comprising a conjugate according to claim 9 and at least one pharmaceutically acceptable carrier or excipient.

26. A composition comprising a conjugate according to claim 15 and at least one pharmaceutically acceptable carrier or excipient.

27. A composition comprising a conjugate according to claim 18 and at least one pharmaceutically acceptable carrier or excipient.

28. A method of treating an infertile mammal, comprising administering to a mammal in need thereof an effective amount of a conjugate according to claim 1.

29. A method of treating an infertile mammal, comprising administering to a mammal in need thereof an effective amount of a conjugate according to claim 9.

30. A method of treating an infertile mammal, comprising administering to a mammal in need thereof an effective amount of a conjugate according to claim 15.

31. A method of treating an infertile mammal, comprising administering to a mammal in need thereof an effective amount of a conjugate according to claim 18.

32. A modified FSH-α polypeptide subunit having an amino acid sequence that differs from that of the wildtype

hFSH-α subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced.

33. A modified FSH-β polypeptide subunit having has an amino acid sequence that differs from that of the wildtype hFSH-β subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced.

34. A nucleotide sequence encoding a modified FSH-α polypeptide subunit having an amino acid sequence that differs from that of the wildtype hFSH-α subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced; and/or encoding a modified FSH-β polypeptide subunit having has an amino acid sequence that differs from that of the wildtype hFSH-β subunit in that at least one amino acid residue comprising an attachment group for a non-polypeptide moiety has been introduced.

35. An expression vector comprising a nucleotide sequence according to claim 34.

36. A host cell comprising a nucleotide sequence according to claim 34.

37. A method for producing a recombinant heterodimeric FSH protein, comprising subjecting a host cell according to claim 34 comprising a nucleotide sequence encoding an FSH-α subunit and an FSH-β subunit to cultivation under conditions conducive for expression of said subunits.

38. The method of claim 37, wherein the host cell is a eukaryotic cell capable of in vivo glycosylation, and the amino acid sequence of at least one of said FSH-α and FSH-β subunits differs from the sequence of the corresponding wildtype subunit in that at least one N-glycosylation site has been introduced.

39. The method of claim 38, further comprising subjecting the heterodimeric protein to in vitro conjugation to a non-polypeptide moiety.

* * * * *

US 20030124737A1

(54) **DIAGNOSTIC KIT FOR PREDICTING PREGNANCY OUTCOME**

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Publication Classification

(57) **ABSTRACT**

The present invention provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample. The present invention further provides a method for determining the amount of early pregnancy associated molecular isoforms of human chorionic gonadotropin (hCG) in a sample. The present invention also provides a diagnostic kit for determining the amount of early pregnancy associated hCG is a sample. The present invention additionally provides an antibody which specifically binds to an early pregnancy associated molecular isoform of human chorionic gonadotropin. Finally, the present invention provides methods for detecting trophoblast or non-trophoblast malignancy in a sample.

FIG. 1

FIG. 2B

FIG. 2A

non-nicked hCG (B109-B109*)
hCG (B152-B207*)
nicked hCG (B151-B207*)
free beta subunit hCG (B201-C104*)
beta-fragment hCG (B210-B108*)

FIG. 3A B601
FIG. 3B B606
FIG. 3C B603
FIG. 3D B605
FIG. 3E B604
FIG. 3F B607
FIG. 3G B610
FIG. 3H B151
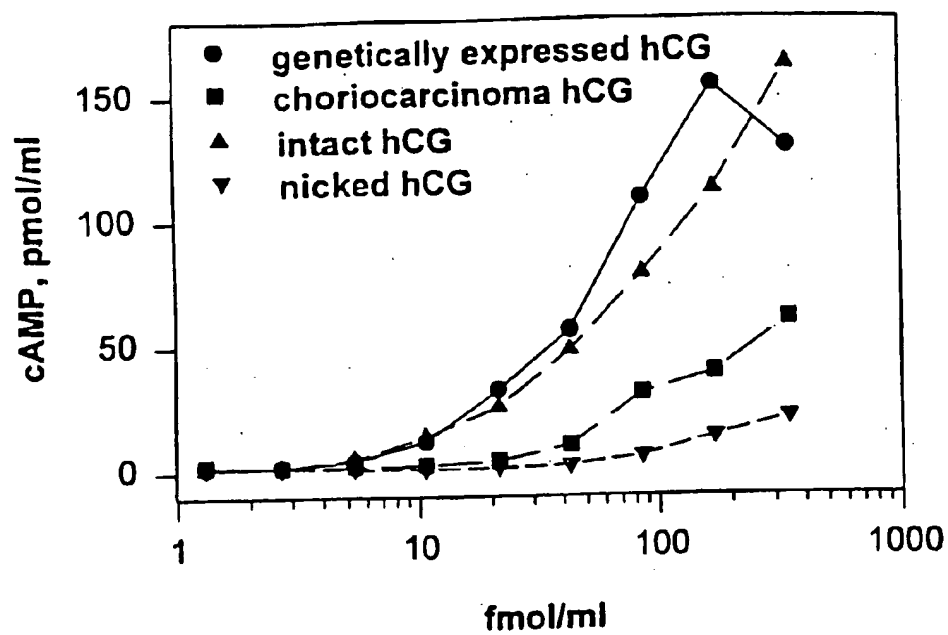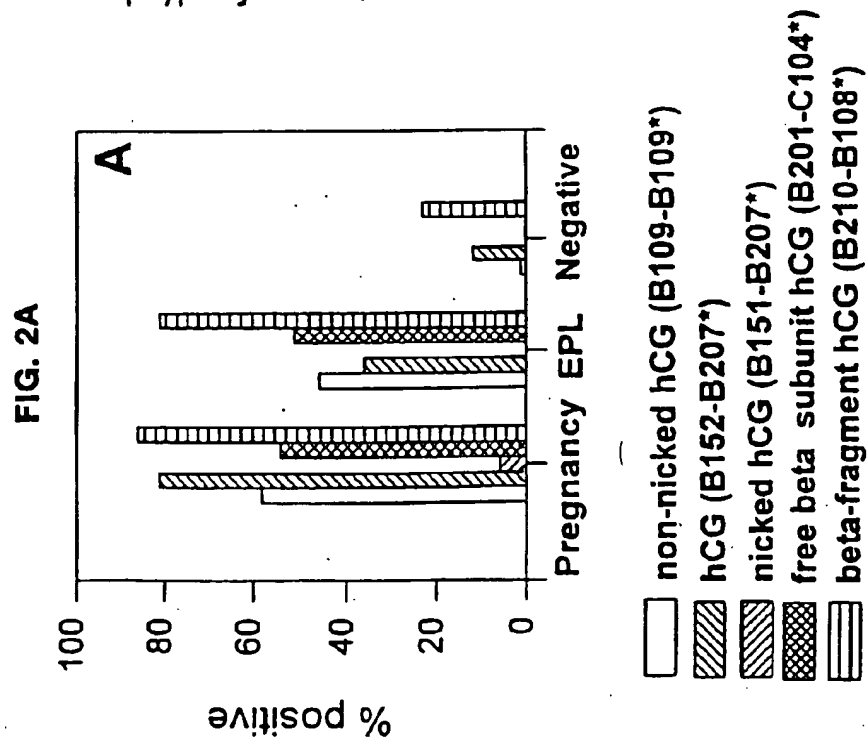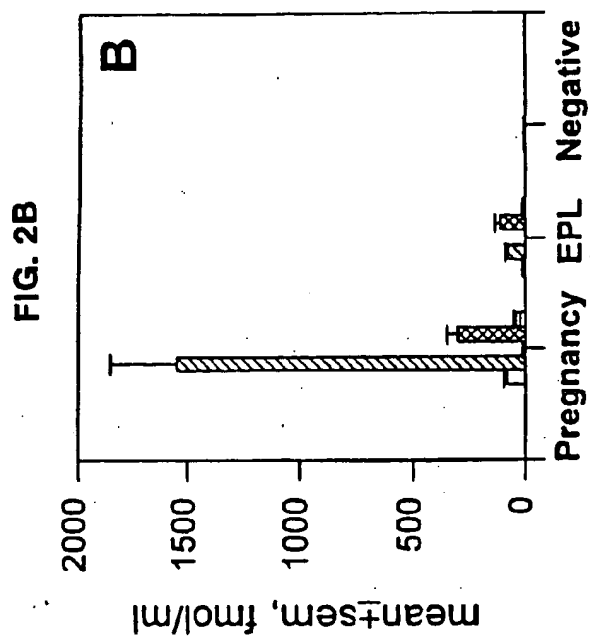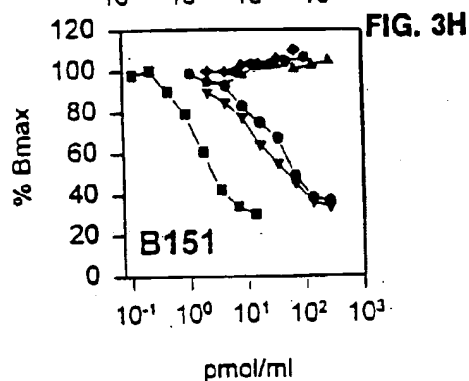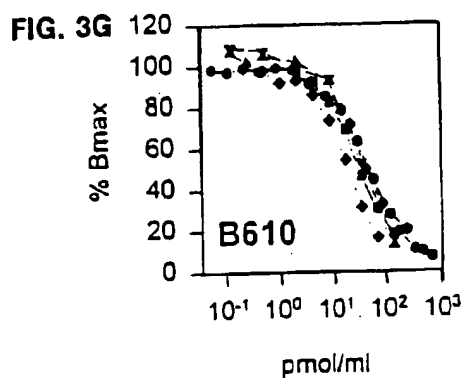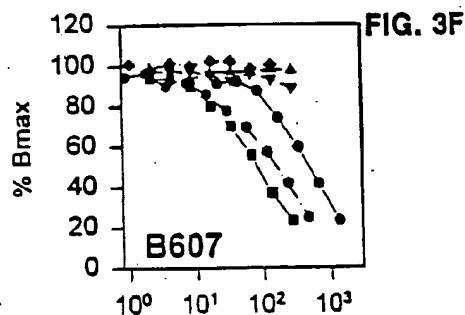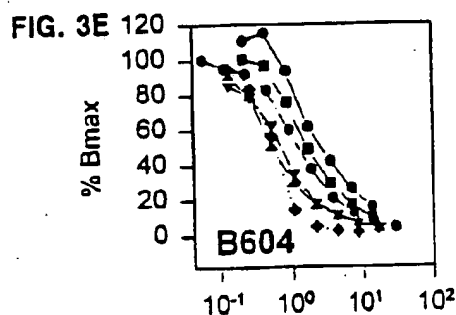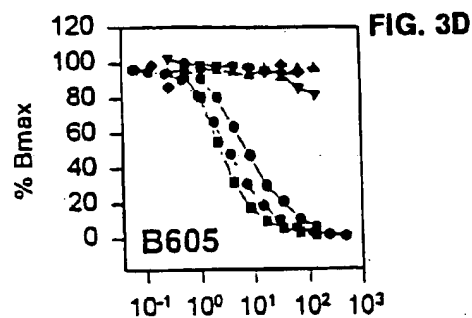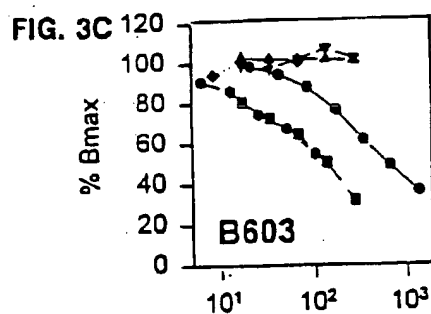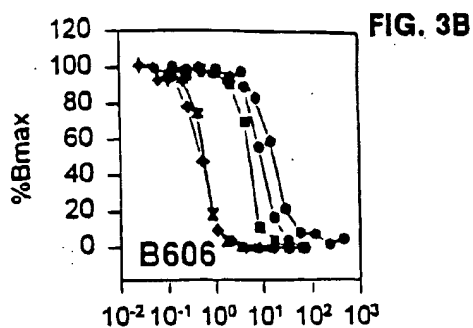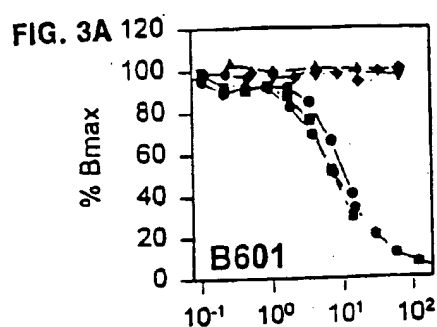
pmol/ml

● hCG   ■ nhCG   ▲ hCGβ   ▼ nhCGβ   ◆ hCGβcf

FIG. 4B
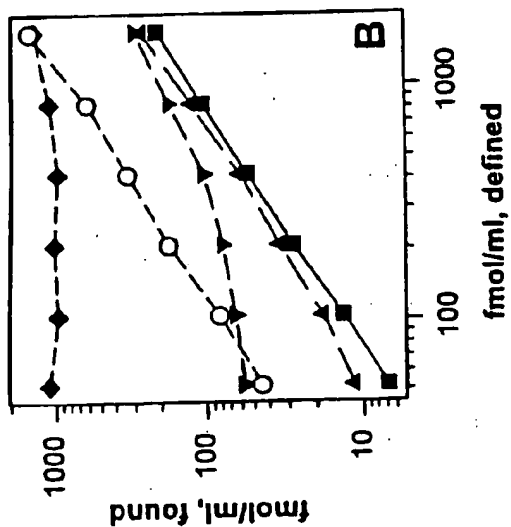
FIG. 4A

FIG. 5B

FIG. 5A
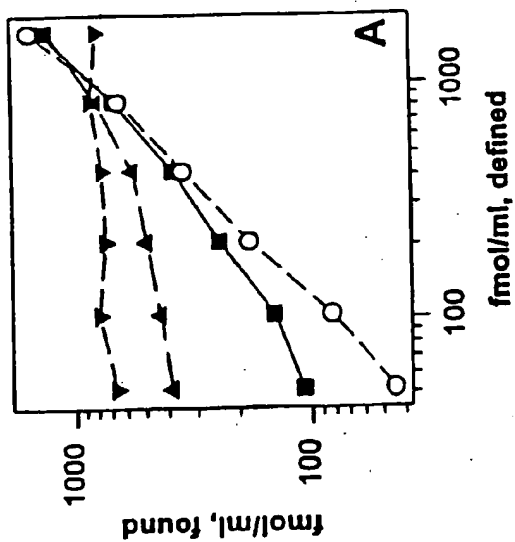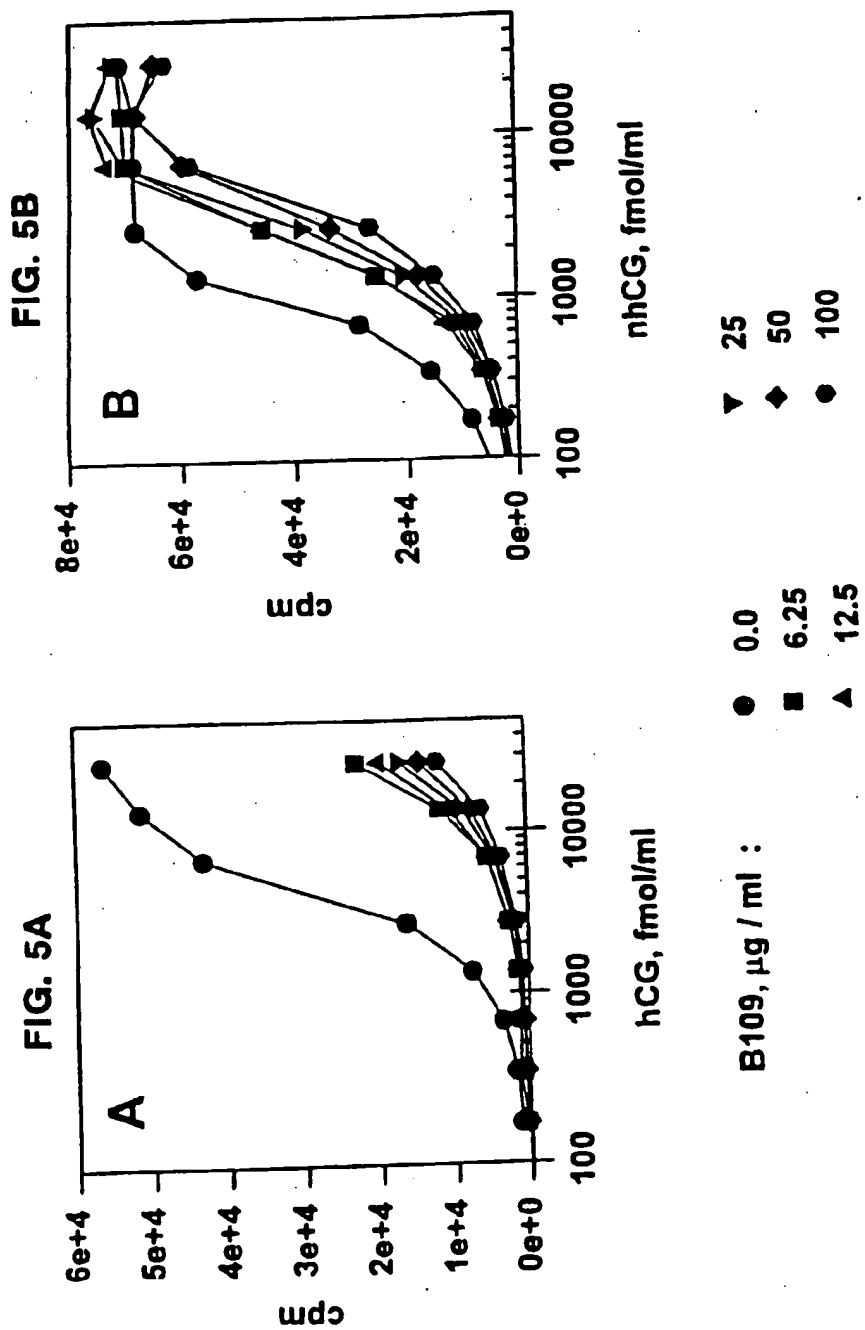
FIG. 6
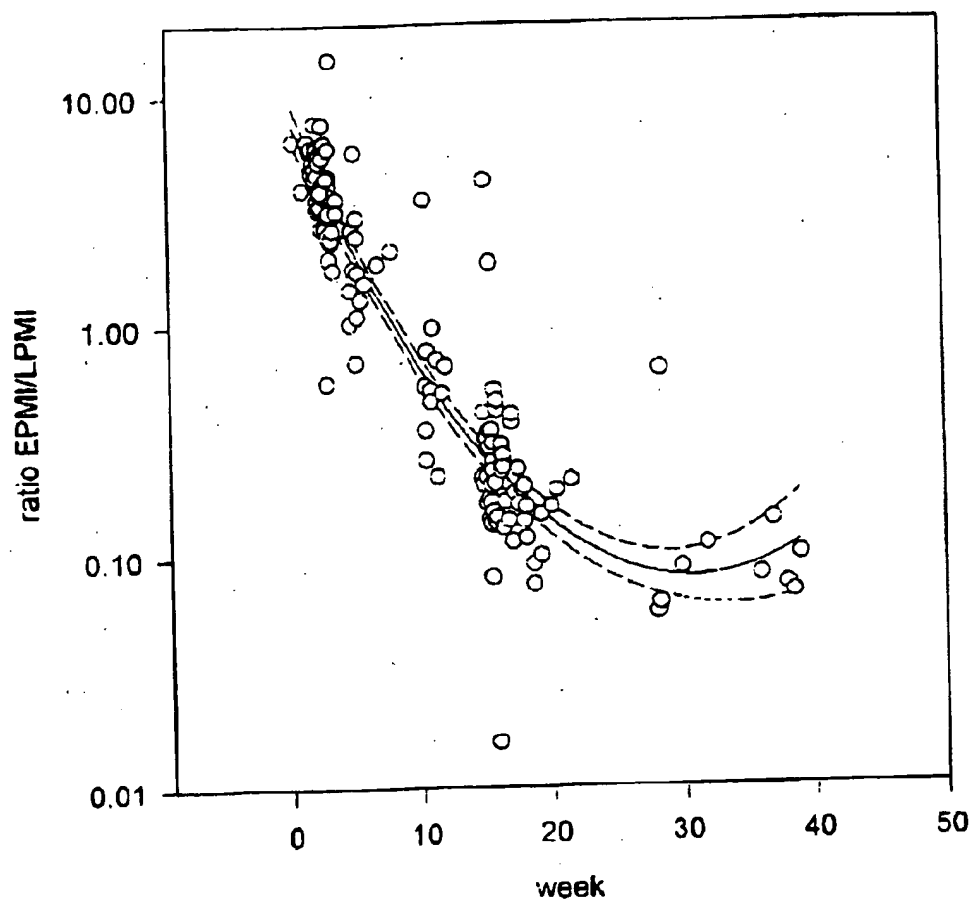
FIG. 7
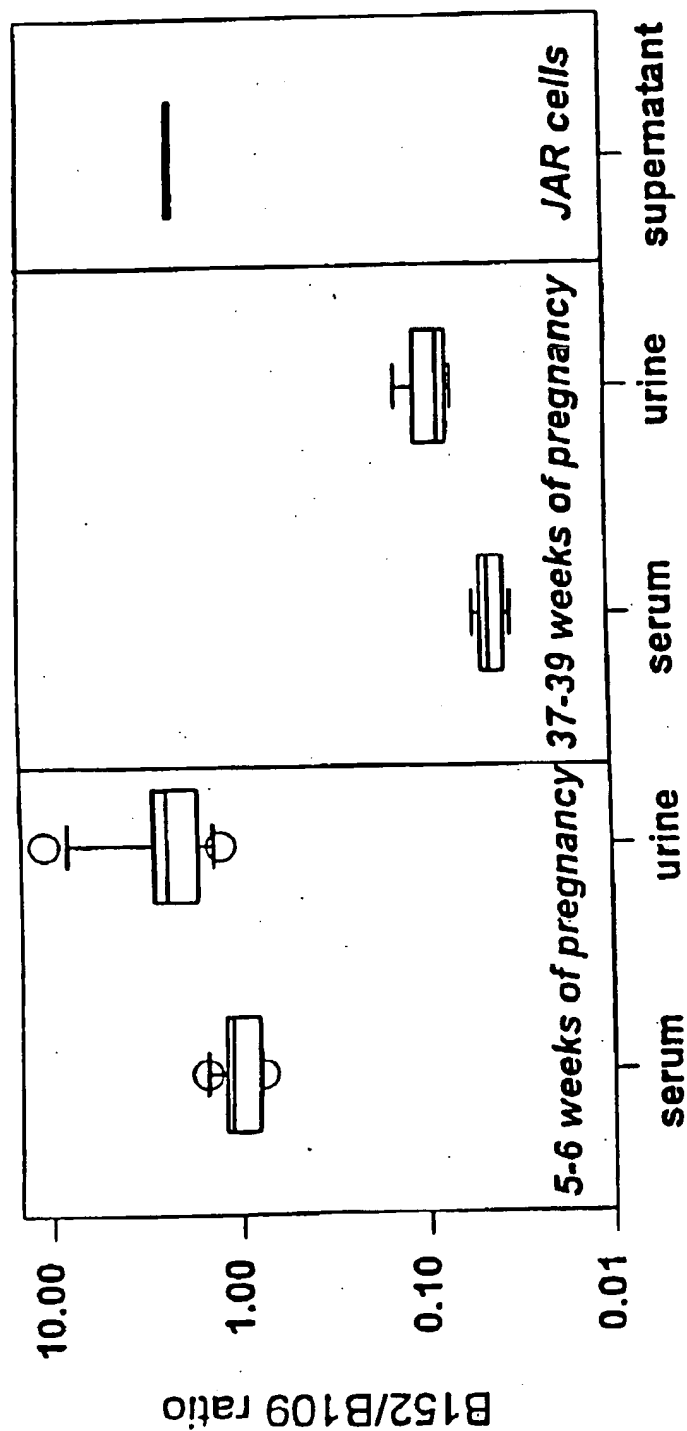
FIG. 8A
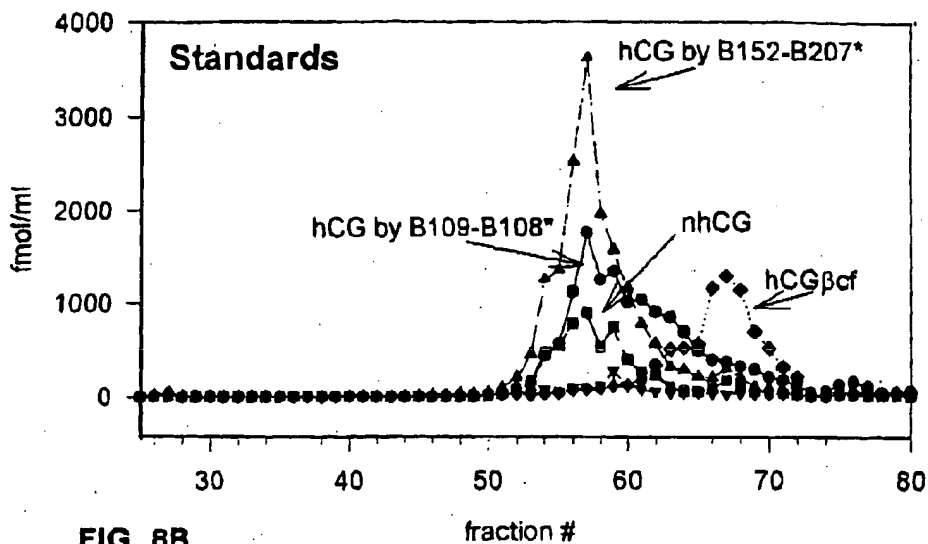
FIG. 8B

FIG. 9

Normal pregnancy vs spontaneous abortion

EPMI hCG

FIG. 10B

LPMI hCG
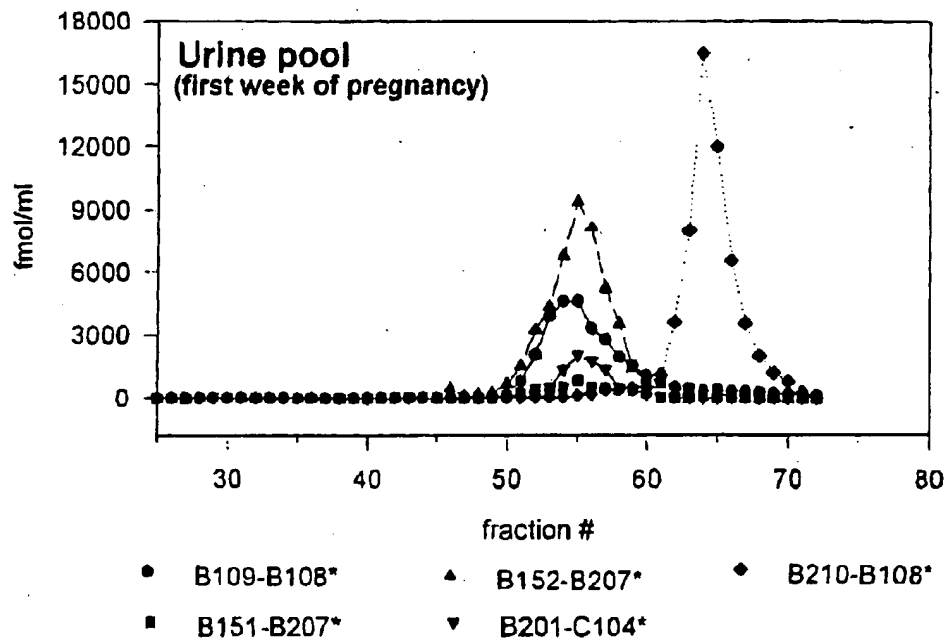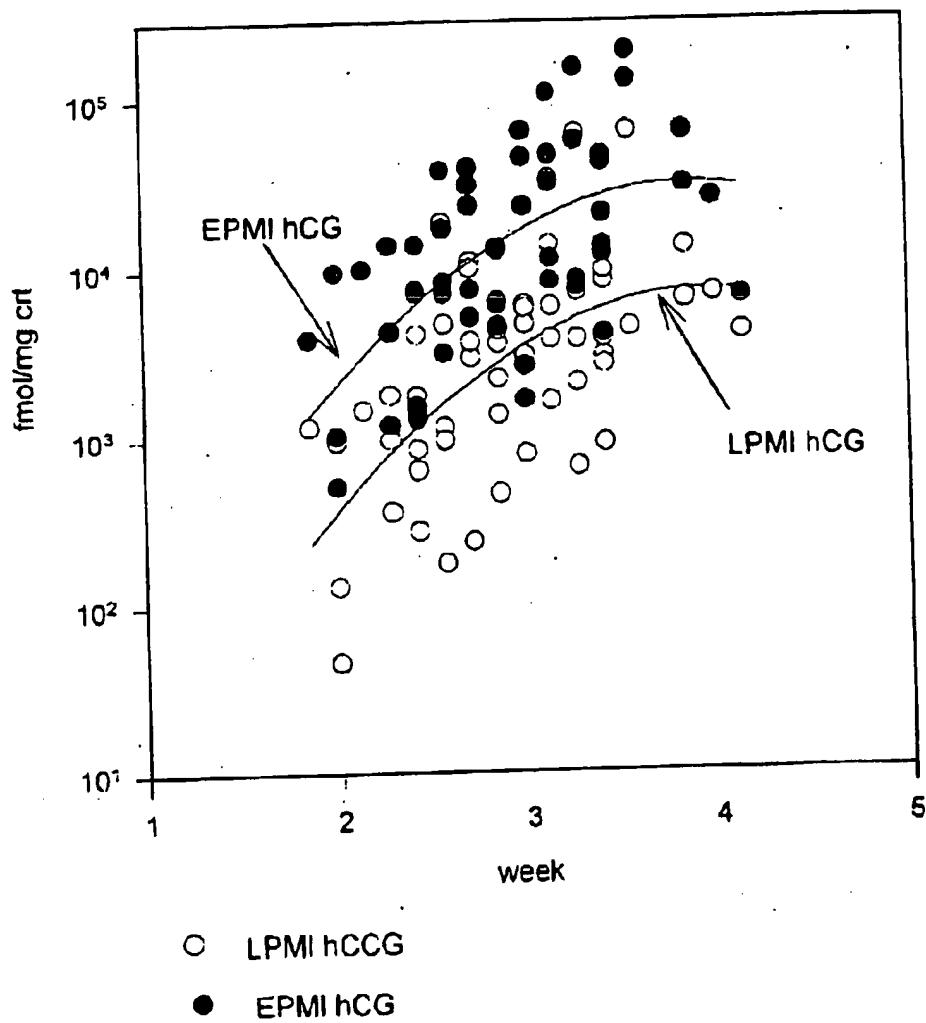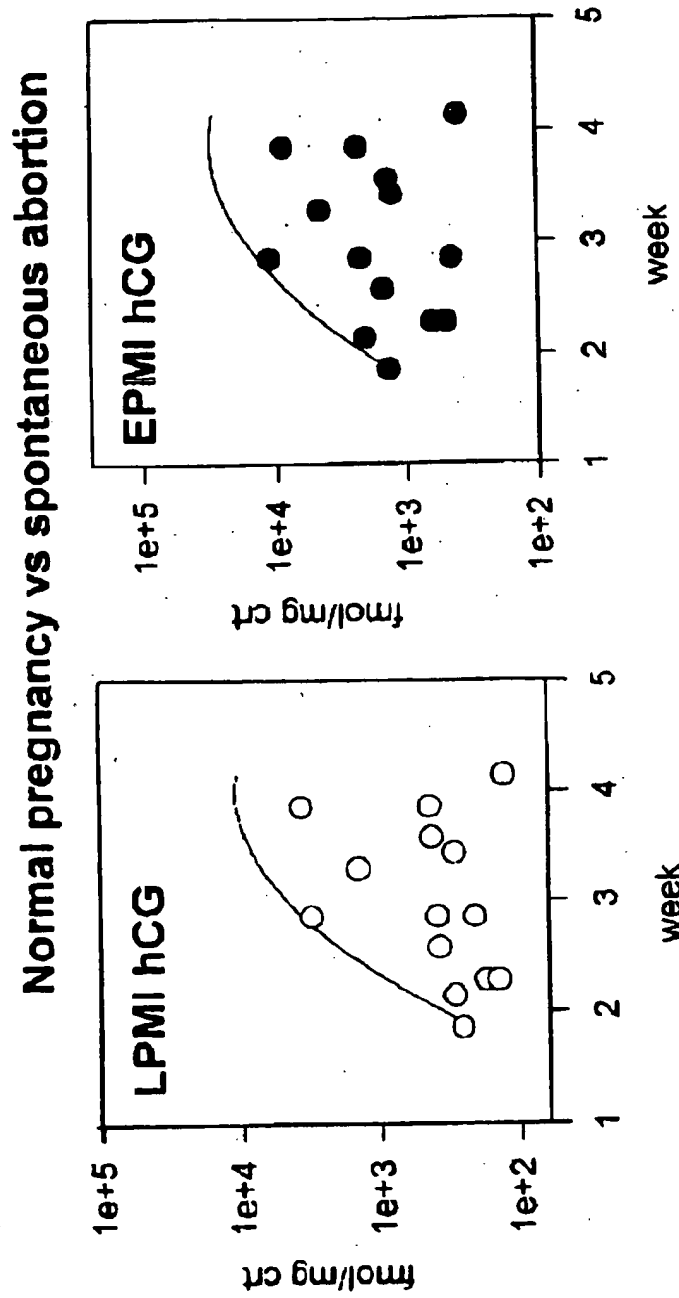
FIG. 10A

Normal pregnancy vs ectopic pregnancy
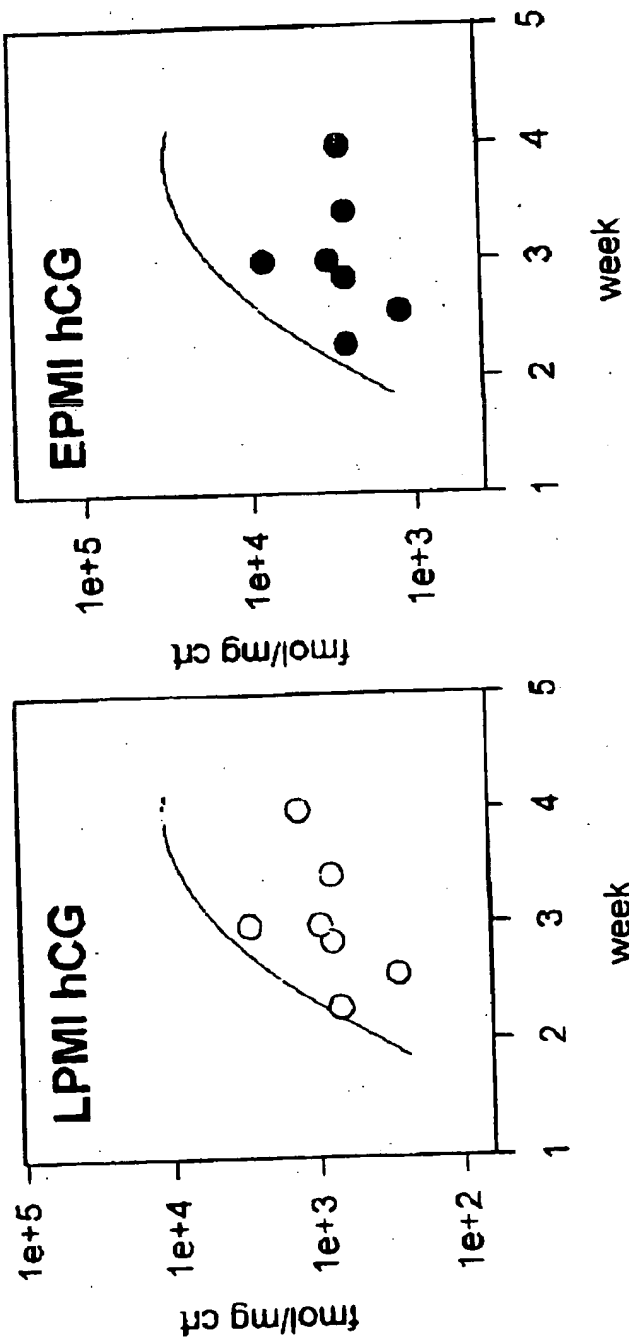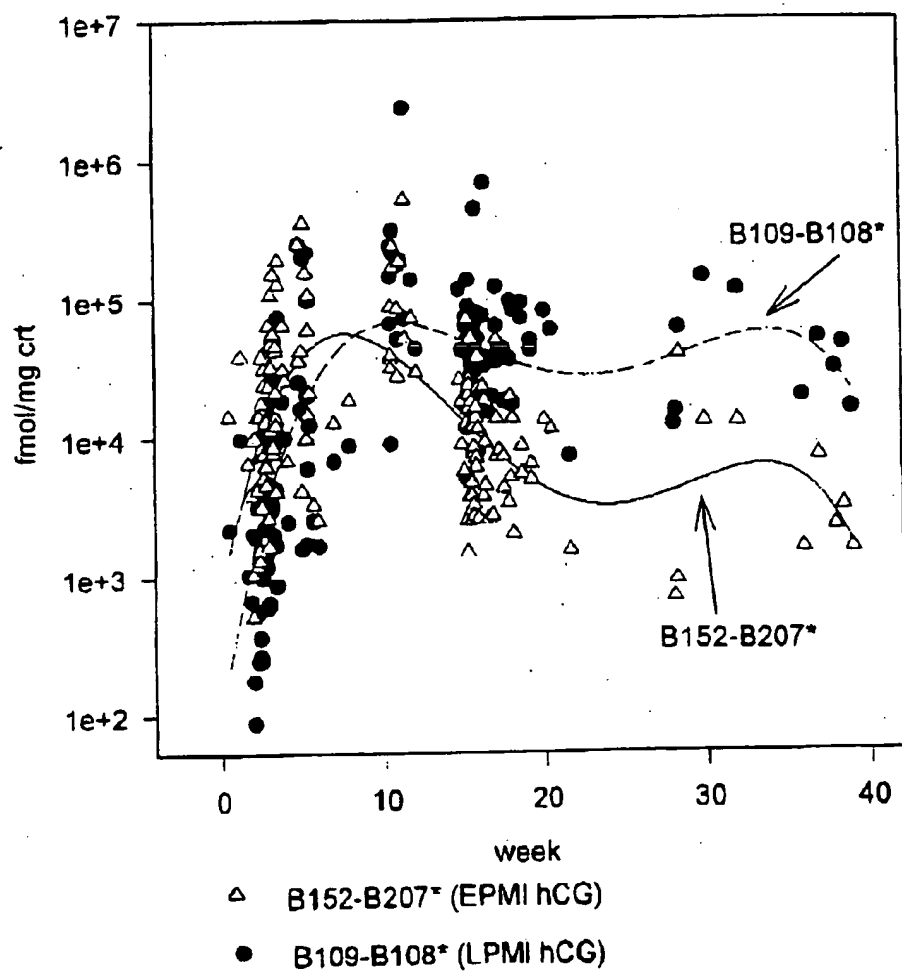
LPMI hCG

1e+5

1e+4

1e+3

1e+2

fmol/mg cf

1   2   3   4   5
week

FIG. 10C

EPMI hCG

1e+5

1e+4

1e+3

fmol/mg cf

1   2   3   4   5
week

FIG. 10D

FIG. 11

DIAGNOSTIC KIT FOR PREDICTING PREGNANCY OUTCOME

BACKGROUND OF THE INVENTION

[0002] Throughout this application, various publications are referenced by author and date. Full citations for these publications may be found listed alphabetically at the end of the specification immediately preceding the claims. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the state of the art.

[0003] Early pregnancy loss (EPL) is a widespread, but largely undiagnosed problem. In order to adequately diagnose and develop treatments for EPL it is essential to be able to detect and measure the rate of occurance of EPL. This is critically important in epidemiological studies, some of which are related to exposures to known or suspected reproductive toxins in the workplace, in the environment or by personal use. These early pregnancy losses are often not recognized by women or physicians and are detected solely by the measurement of hCG in the urine at the time between implantation and expected menses. They are sometimes termed "chemical pregnancies" or "occult pregnancies." A landmark epidemiological study established that the incidence of EPL was 22% in a population of healthy women attempting to conceive (Wilcox, A. J., et al., 1988). This investigation employed a very sensitive (0.01 ng/ml hCG) assay which detected only the intact hCG molecule with the unique beta subunit carboxyterminal peptide present.

[0004] There are multiple likely causes for EPL and clinical spontaneous abortion including genetic abnormality, immunological dysfunction, untreated infection or other unknown physiological problems. In addition, losses may be caused by failure of human chorionic gonadotropin (hCG) to induce adequate response at its target, the corpus luteum.

[0005] This could result from inadequate hormonal potency.

[0006] "Nicking" of the beta subunit in the loop 2 region of the molecule, specifically between residues 44-49, can reduce bioptency of hCG. Cleaved peptide bonds in this area of the molecule also exhibit reduced biopotency and reduced immunochemical recognition by monoclonal antibodies directed to the heterodimeric hormone (Cole, L. A., et al., 1991a; Cole, L. A., et al., 1991b; Puisieux, A., et al., 1990; Nishimura, R., et al., 1988; Nishimura, R. T., et al., 1989). Nicked forms of hCG were examined as possibly more prevalent in EPL situations and, at least in part responsible, for early pregnancy loss. Unfortunately many of the reports claiming that substantial concentrations of nicked hCG are produced during pregnancy, losses or successful pregnancies, are not accurate due to faulty assumptions regarding assay specificity (Wilcox, A. J., et al., 1988). Carbohydrate-modified hCG can also exhibit reduced biopotency. It is known that if the hCG has much reduced sialic acid content and its carbohydrate chains terminate in galactose, much hCG would be removed by the liver receptor for such altered

glycoproteins (Braun, J. R., et al., 1996; Kawasaki, T. and G. Ashwell, 1996). The circulating life-time of asialo hCG is reduced and its in vivo potency is thereby low. Other carbohydrate changes also alter circulating half life; glycoproteins terminating in sulfate-N-acetyl galactosamine are also extracted by a specific liver receptor and have reduced circulating lifetime (Baenziger, J. U., 1994; Fiete, D., et al., 1991).

[0007] At least two factors affect increased potency of hCG. First, it is known that a larger Stoke's radius will decrease clearance through the kidney glomerulus which generally clears proteins above an effective size of 70,000 very slowly. The effective size of urinary-isolated hCG is just at this borderline reduced clearance size. Generally, extra sugar content makes the hydrated radius of glycoproteins larger. It has been shown that by adding the hCG beta COOH-terminal peptide to hFSH or hLH, their circulating life-times greatly increased (Fares, F. A. et al., 1992; Matzuk, M. M., 1990). This addition was thought mostly due to the carbohydrate content of that peptide rather than simply the extra polypeptide size (Wilcox, A. J., et al., 1988). Second, increased negative charge of a protein will prolong its circulating time because of decreased renal clearance (Chmielewski, C. 1992, Quadri, K. H., et al., 1994; Maack, T., et al., 1985). This increased negative charge can be due to extra sialic acid or other negative groups, including sulfate such as is present on hLH and on the pituitary form of hCG (Birken, S., et al., 1996b). Changes which affect signal transduction at the receptor may also affect biopotency of hCG. It is known that deglycosylated hCG has much reduced receptor potency (Ravindranath, N., et al., 1992; Sairam, M. R., and L. G., Jiang, 1992; Browne, E. S., et al., 1990; Sairam, M. R., 1989; Sairam, M. R., et al., 1988). Carbohydrate reduced forms of hCG also have reduced signal transduction (Amano, J., et al., 1990; Bahl, O. P., et al., 1995; Moyle, W. R., 1975).

[0008] According to the present invention EPL or recurrent spontaneous abortion is not due to an abnormal hCG form that has reduced potency, such as nicked hCG. Instead, the present invention provides evidence that in successful outcome pregnancies women usually produce forms of hCG which are very highly potent in very early pregnancy; the standard urinary reference preparations of hCG are less potent forms of the hormone produced later in pregnancy. The increased potency could be caused by a combination of factors from circulating half-life to increased receptor affinity or signal transduction or all of the preceding. Since hCG is low very early in pregnancy, it is logical to find a more potent form of hCG on a molar basis to carry out its function until production levels rise as the trophoblastic cellular mass increases. The present invention describes molecular and immunological tools and methods including an antibody, B152, described herein which recognizes the highly potent early pregnancy associated molecular isoforms of hCG.

[0009] The determination of blood and urine profiles for the B152 hCG isoforms throughout healthy pregnancies can delineate the pattern of isoforms in successful pregnancies. These isoforms can be measured by immunoassay alone, obviating the need to perform complex isoelectric focusing studies or other separation techniques. Additionally, the methods decribed herein are applicable to large numbers of samples.

SUMMARY OF THE INVENTION

[0010] The present invention provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (c) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject.

[0011] The present invention further provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a capturing antibody which specifically binds to the early pregnancy associated molecular isoform of hCG with a solid matrix under conditions permitting binding of the antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody; (c) separating the bound matrix and the sample; (d) contacting the separated bound matrix with a detecting antibody which specifically binds to hCG under conditions permitting binding of antibody and antigen in the sample; (e) measuring the amount of bound antibody on the bound matrix, thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample; and (f) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (e) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

[0012] In addition, the present invention provides a method for determining the amount of early pregnancy associated molecular isoforms of in a sample comprising: (a) contacting the sample with an antibody which specifically binds to an early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; and (b) determining the amount of complexes formed thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample.

[0013] Further, the present invention provides a diagnostic kit for determining the amount of early pregnancy associated

hCG is a sample comprising: (a) an antibody which specifically binds to an early pregnancy associated molecular isoform; (b) a solid matrix to which the antibody is bound; and (c) reagents permitting the formation of a complex between the antibody and a sample.

[0014] The present invention additionally provides an antibody which specifically binds to an early pregnancy associated molecular isoform of human chorionic gonadotropin.

[0015] Further, the present invention provides a method for detecting non-trophoblast malignancy in a sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (d) comparing the amount of early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with the amount of early pregnancy associated molecular isoform of hCG in the sample determined in step (c), wherein a positive detection of early pregnancy associated molecular isoform detected in step (b) and a relative absence of the early pregnancy associated molecular isoform of hCG detected in step (c) indicates the presence of non-trophoblast malignancy in the sample.

[0016] Finally, the present invention provides a method for detecting gestational trophoblast disease in a sample from a subject comprising (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample due to binding with the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody; (d) determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the subject; and (e) comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

BRIEF DESCRIPTION OF THE FIGURES

[0017] FIG. 1

[0018] Bioassay for forms of hCG. This is data from recombinant CHO cells expressing the LH/CG receptor. The response factor is cAMP production. The x-axis is dose of one of four calibrated, pure hormones as described on graph legends. Expressed hCG has no nicks; choriocarcinoma hCG (C5) is 100% nicked; CR 127 was purified into a nick-free (non-nicked, intact) and nick-enriched fraction as shown.

[0019] FIGS. 2A-2B

[0020] The percentage of positive samples (FIG. 2A) in the study for each of the analytes measured (In early normal pregnancy, EPL cycles and control, i.e. non-conceptive cycles. It is apparent that all of the characterized hCG urinary analytes, except nicked hCG, are expressed in both pregnancy and EPL. In the non-conceptive (control) cycles hCG beta core fragment is expressed at low levels in about 20% of the cycles FIG. 2B provides the levels of hCG analytes expression in the three types of cycles denominated above. Clearly, the pregnancy cycles produced greater amounts of analytes that did the loss cycles, with the most obvious difference being provided by the B152-B207* hCG assay. (Specimens were collected at days 9, 10, 11 post calculated day of ovulation.

[0021] FIGS. 3A-3H

[0022] Specificity characteristics of monoclonal antibodies to nicked hCG in liquid phase competitive assays.

[0023] FIGS. 4A-4B

[0024] Recovery nhCG in B151-B604* assay in the presence of increasing concentrations of hCG (FIG. 4A) and in the presence of B109 (0.01 mg/ml) as a scavenger for hCG (FIG. 4B).

[0025] FIGS. 5A-5B

[0026] Effect of different concentrations of B109 as a scavenger for hCG in B115-B108* assay (FIG. 5A) and effect of B109 in different concentrations on nicked hCG binding in B151-B108* assay (FIG. 5B).

[0027] FIG. 6

[0028] Ratio of hCG isoforms measured by the B152-B207* (early pregnancy associated molecular isoform (EPMI) hCG) and B109-B108* (late pregnancy associated molecular isoform (LPMI) hCG) assays in normal pregnancy urine (n=159) as a function of gestational age. (The second order regression curve and 95% confidence intervals are shown).

[0029] FIG. 7

[0030] Box plot of B152/B109 ratio (EPML/LPMI hCG) for pregnancy matched serum/urine specimens at 5-6 weeks of gestational age (n=5) and in JAR cell supernatant. Box extends to the 25th and 75th percentile. The upper and lower symbols indicate the 90th and 10th percentile respectively. A solid line inside the box marks the value of the 50th percentile. The ratio in JAR supernatant, choriocarcinoma cell line, is similar that in early pregnancy, i.e. B152-B207* may be more sensitive marker for the type of hCG produced in malignancy.

[0031] FIGS. 8A-8B

[0032] FIG. 8A. Dual tandem Superose 12 columns of standard preparation of intact hCG, nicked hCG and hCG beta core fragment as assessed by specific immunoassays.

[0033] FIG. 8B. Dual tandem Superose 12 columns of a pooled early pregnancy urine concentrate (first week of gestation), illustrating that there is no additional signal for molecules of lower molecular size.

[0034] FIG. 9

[0035] Urinary concentration of EPMI hCG and LPMI hCG in the first 4 weeks of gestation (n=57). (The second order regression curve and molar concentrations values are shown).

[0036] FIGS. 10A-10D

[0037] Urinary concentration of EPMI and LPMI hCG in spontaneous abortion (n=14) and ectopic pregnancy (n=7) against matched gestational age normal pregnancy samples (n=57) (for normal pregnancy only regression curves shown). There is a statistically significant concentration difference between normal and ectopic pregnancy or spontaneous abortion both EPMI and LPMI. They both discriminate well on the basis of concentration.

[0038] FIG. 11

[0039] The urinary intact hCG profile expressed in fmol/mg creatinine throughout the pregnancy as measured by B109-B108* assay (LPMI hCG) and by B152-B207* assay (EPMI hCG) (n=159). EPMI appears earlier in gestation and decreases more substantially than does LPMI as pregnancy progresses.

DETAILED DESCRIPTION OF THE INVENTION

[0040] A method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (c) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject. In an embodiment of the present invention, the antibody is B152. Another embodiment of this invention is the early pregnancy associated molecular isoform of hCG.

[0041] According to one embodiment of this invention, step (a) further comprises a second antibody which specifically binds to hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. In an embodiment of

this invention, the second antibody is B207. According to another embodiment of this invention, step (a) further comprises a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. In an embodiment of this invention, the second antibody is B108 or B109. In an embodiment of this invention, step (c) comprises comparing the amount of the early pregnancy associated molecular isoform of hCG determined in step (b) for said antibody with the amount determined in step (b) for the second antibody, wherein a high ratio of amounts determined for said antibody relative to the second antibody indicates a positive outcome of pregnancy for the subject, a low ratio indicates a negative outcome of pregnancy for the subject.

[0042] In yet another embodiment of this invention, step (c) comprises comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

[0043] According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125}.

[0044] The present invention further provides a method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising: (a) contacting a capturing antibody which specifically binds to the early pregnancy associated molecular isoform of hCG with a solid matrix under conditions permitting binding of the antibody with the solid matrix; (b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody; (c) separating the bound matrix and the sample; (d) contacting the separated bound matrix with a detecting antibody which specifically binds to hCG under conditions permitting binding of antibody and antigen in the sample; (e) measuring the amount of bound antibody on the bound matrix, thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample; and (f) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (e) with either (i) the amount determined for temporally matched, normal preg-

nant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

[0045] An embodiment of this invention further comprises (a) removing of the sample from the matrix; and (b) washing the bound matrix with an appropriate buffer. In one embodiment of this invention, the capturing antibody is B152. In one embodiment of this invention, the detecting antibody is B207. In an embodiment of this invention, step (a) further comprises a second capturing antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. According to an embodiment of this invention, the second capturing antibody is B108 or B109. In an embodiment of this invention, step (d) further comprises a second detecting antibody which specifically binds to hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG. In an embodiment of this invention, step (f) comprises comparing the amount of the early pregnancy associated molecular isoform of hCG determined in step (e) for said antibody with the amount determined in step (b) for the second antibody, wherein a high ratio of amounts determined for said antibody relative to the second antibody indicates a positive outcome of pregnancy for the subject, a low ratio indicates a negative outcome of pregnancy for the subject.

[0046] According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125}.

[0047] In addition, the present invention provides a method for determining the amount of early pregnancy associated molecular isoforms of in a sample comprising: (a) contacting the sample with an antibody which specifically binds to an early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; and (b) determining the amount of complexes formed thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample.

[0048] According to an embodiment of this invention, the antibody specifically binds a region of the early pregnancy associated molecular isoform of hCG comprising a carbo-

hydrate moiety. In one embodiment of this invention the antibody is produced by a hybridoma cell line. In one embodiment of this invention the antibody is B152.

[0049] Further, the present invention provides a diagnostic kit for determining the amount of early pregnancy associated hCG is a sample comprising: (a) an antibody which specifically binds to an early pregnancy associated molecular isoform; (b) a solid matrix to which the antibody is bound; and (c) reagents permitting the formation of a complex between the antibody and a sample. In an embodiment of this invention, the antibody is B108, B109 or B152. An embodiment of this invention further comprises control sample(s) normal pregnant sample(s), nonpregnant sample(s), or male sample(s).

[0050] According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125}.

[0051] The present invention additionally provides an antibody which specifically binds to an early pregnancy associated molecular isoform of human chorionic gonadotropin.

[0052] In an embodiment of this invention, the antibody specifically binds to a region of the early pregnancy associated molecular isoform of human chorionic gonadotropin comprising a carbohydrate moiety. According to one embodiment of this invention, the monoclonal antibody is B152. In an embodiment of this invention, a hybridoma cell (ATCC Accession No. _____) is provided capable of producing monoclonal antibody B152. Another embodiment of this invention is the early pregnancy associated molecular isoform of hCG recognized by the B152 monoclonal antibody.

[0053] Further, the present invention provides a method for detecting non-trophoblast malignancy in a sample comprising: (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and (d) comparing the amount of early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with the amount of early pregnancy associated molecular isoform of hCG in the sample determined in step (c), wherein a positive detection of early pregnancy associated molecular isoform detected in step (b) and a relative absence of the early pregnancy

associated molecular isoform of hCG detected in step (c) indicates the presence of non-trophoblast malignancy in the sample.

[0054] According to an embodiment of this invention, the antibody is B604, B151, B152 or B207. In an embodiment of this invention, the second antibody is B108, B109. In an embodiment of this invention, the non-trophoblast malignancy is ovarian malignancy or prostate malignancy.

[0055] According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125}.

[0056] Finally, the present invention provides a method for detecting gestational trophoblast disease in a sample from a subject comprising (a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; (c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample due to binding with the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody; (d) determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the subject; and (e) comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

[0057] In an embodiment of this invention, the antibody is B604, B151, B152 or B207. In another embodiment of this invention, the second antibody is B108, B109. In an embodiment of the present invention, the gestational trophoblast disease is choriocarcinoma or hydatidiform mole.

[0058] According to an embodiment of this invention, the sample is a urinary sample or a blood sample. In one embodiment of this invention, the sample is an aggregate sample taken from at least two consecutive days. In an embodiment of this invention, the sample is a spot urine sample, a first morning void urine sample, or an aggregate sample of the first morning void urine samples for at least two consecutive days. In one embodiment of this invention, the antibody is labeled with a detectable marker. In an

embodiment of this invention, the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin. In a preferred embodiment, the radioactive isotope is I^{125}.

[0059] As described herein below, unexpected isoforms of hCG are produced during normal early pregnancy. Using an in vitro bioassay, it appears that these isoforms have enhanced potency for signal transduction. These isoforms can be measured using the novel sensitive, immunoassay described herein. This can help predict pregnancy outcome where one cause of early pregnancy loss is failure to produce the isoform of hCG of higher potency produced by success- ful pregnancies. This enables physicians to intervene to sustain a failing pregnancy. Identification of the nature of the hCG isoform required might provide the proper reagent needed to sustain pregnancy.

[0060] New antibodies for measurement of nicked forms of hCG described herein below were developed based on the hypothesis that forms of hCG, which have greatly reduced bioactivity, contribute to early pregnancy loss (EPL), due at least in part to diminished biopotency. Evidence was found that the hCG that appears in EPL patients displays reduced biological activity. However, it was determined that the cause of the reduced bioactivity is not the presence of nicked hCG in EPL patients. Instead, the hypothesis is that patients that carry pregnancies forward produce an isoform of hCG with enhanced bioactivity. The instant invention describes a unique immunochemical assay to measure this unexpected and previously uncharacterized isoform of early pregnancy hCG directly in clinical samples of blood and urine. One of the antibodies developed reacted against a nicked form of hCG isolated from a choriocarcinoma patient, was not specific for a nicked form of hCG but appeared to discrimi- nate among carbohydrate variants of hCG. This antibody, designated B152, appears to preferentially bind hCG forms from choriocarcinoma patients. In studying the content of hCG isoforms during pregnancy, the unique and unexpected observation was made that B152 in the first four weeks of pregnancy measured much higher quantities of an isoform of hCG as compared to the standard hCG isoforms measured by the usual heterodimeric hCG assays exemplified by a previously decribed B109 based assay. In fact, in early pregnancy (days 9, 10, 11 postovulation) B152 measured as much as 20-fold more hCG, than did another monoclonal antibody, B109. Later in pregnancy, the B152 isoform declines and is lower in third trimester pregnancy urine than the standard isoforms measured by B109. A further striking observation was that in very early pregnancy, a high B152/ B109 ratio correlates with a successful pregnancy outcome while a low ratio correlated with pregnancy loss. This discovery is important as the potentially overlooked iso- forms of hCG described herein during pregnancy may be predictors of successful pregnancy outcome. Such an assay has wide medical applications and provides a clinician with opportunity to intervene very early in pregnancy if the assay indicated that the pregnancy appeared troubled.

[0061] An antibody, designated B152, produced by the hybridoma cell accorded ATCC Accession number _____, generated against a nicked form of hCG isolated from a choriocarcinoma patient, but not specific for nicked isoform hCG is able to discriminate among carbohydrate variants of hCG. B152 is specific for an early pregnancy associated molecular isoform of hCG which in the first four weeks of pregnancy is measured at much higher quantities than the

hCG standard isoforms measured by the usual heterodimeric hCG assays exemplified by a previously decribed B109 based assay. Later in pregnancy, the B152 isoform declines and is lower in third trimester pregnancy urine than the standard isoforms measured by B109.

[0062] Antibodies specific for hLH beta core fragment some of which are referred to in the present application, have been detailed in the related co-pending U.S. application Ser. No. 08/763,669 filed Dec. 11, 1996, the content of which is hereby incorporated by reference. In particular, related co-pending U.S. application Ser. No. 08/763,669 filed Dec. 11, 1996, describes monoclonal antibody desig- nated B505 which is produced by the hybridoma cell line accorded ATCC Accession No. 12000 and details methods for its production and use, which is hereby incorporated by reference.

[0063] This invention is illustrated in the Experimental Details section which follows. These sections are set forth to aid in an understanding of the invention but are not intended to, and should not be construed to, limit in any way the invention as set forth in the claims which follow thereafter.

[0064] Experimental Details

EXAMPLE 1

Antibodies to and Analysis of Molecular Isoforms of hCG in Early Pregnancy

[0065] Introduction

[0066] Almost all investigations of the incidence of early pregnancy loss (EPL), either in normal populations or in- populations at risk as a consequence of exposure to putative reproductive toxins (Hakim, R. B., et al., 1995; Lasley, B. L., et al., 1995) use assays for heterodimeric, non-nicked hCG or combination assays which include free beta subunit and beta core fragment of hCG. One concern about the forms of hCG to include in the measurement in EPL was heightened with respect to the nicking phenomenon described above. Because nicked hCG molecules are not measured by the antibodies employed in most EPL studies, the incidence of EPL is presumably underestimated by an amount propor- tional to the extent of nicking in the urinary molecule. Another concern of significant importance was a determi- nation of the nature of the "hCG like" immunoreactivity in the urine in the periovulatory surge of the menstrual cycle (O'Connor J., et al., 1995). Recent reports have confirmed the existence of and documented the structure of a sulfated form of hCG produced in the pituitary (Birken, S., et al., 1996b). There is a pulsatile secretion of hCG in both men and non-pregnant women. (Odell, W. D.; Griffin, J., 1989 and Odell, W. D.; Griffin, J., 1987). The presence of a non-pregnancy associated form of sulfated hCG of pituitary origin, peaking at ovulation and perhaps persisting into the luteal phase, could potentially interfere with the accurate estimation of EPL.

[0067] Unappreciated isoforms of hCG in blood and urine very early in pregnancy may be more potent in vivo than the forms of hCG produced later in pregnancy. The absence of such isoforms may be one cause of early pregnancy loss. A sensitive and specific immunoassay system was designed and made to measure unique early pregnancy associated molecular isoforms (EPMI) of hCG. These isoforms, likely

to differ by carbohydrate composition, are predictive of a successful pregnancy outcome. When these early pregnancy associated molecular isoforms of hCG are absent or present in low concentration, the pregnancy may be lost very early and be observed as only a "chemical" pregnancy. These hCG isoforms may resemble the forms of hCG produced in some choriocarcinoma patients from which the immunogen used to produce monoclonal antibody B152 was derived as described herein below. The isoforms resemble those from trophoblastic disease not in terms of nicking or intact peptide chains but likely in carbohydrate content. The present invention describes that the molar ratio of B152 to B109 epitopes are predictive of a successful pregnancy or a loss. Three categories of pregnant patients were analyzed: (a) normal pregnant women, (b) women who experience recurrent abortions, (c) women undergoing embryo implantation.

[0068] It is possible to determine the hCG isoforms present in the blood and urine of women who have a history of recurrent spontaneous abortion and a similar analysis of women undergoing embryo implantation. The combined EPL and spontaneous abortion rate in healthy populations is 31%. Subjects who experience three consecutive recurrent spontaneous abortions have a 32% risk of sustaining another (Hill, J. A.; Anderson, D. J., 1990). In in vitro fertilization IVF pregnancy, the loss rate is 70% with non-donor sperm and 50% when donor sperm is used. Delineation of pregnancies with a negative outcome from pregnancies with a positive outcome can be based on differences in the concentrations of EPMI hCG isoforms (i.e. as differences in the B152/B109 ratio in patients). In addition, specimens from gestational trophoblastic disease (GTD) can be used to discriminate between GTD and normal pregnancy.

[0069] Results

[0070] In vitro Bioassay for hLH/hCG

[0071] An hCG bioassay was constructed employing CHO cells expressing functional human LH/CG receptor. FIG. 1 illustrates the differences in vitro in biological activity between nicked and non-nicked hCG as measured by this assay. This system has been used to evaluate the activity of pituitary and placental hCG (Birken, S., et al., 1996b). Preparations of hCG were tested for nicked and non-nicked molecular isoforms of hCG in a second recombinant bioassay system (Ho, H-H., et al., 1997). Similar results were obtained in both systems.

[0072] Normal pregnancy values compared with EPL values. Results indicated that nicked hCG is not a significant molar constituent of either early pregnancy or EPL. Data indicated that biological activity is not correlated with nicked hCG, but is instead ascribed to a form of hCG recognized by the B152 monoclonal antibody—an early pregnancy associated molecular isoform of hCG (EPMI hCG). It has been established that there is diminished hCG bioactivity associated with EPL as compared to early normal pregnancy (Ho, H-H., et al., 1997). Thus, diminished hCG biological activity is a factor in EPL as a consequence of a heretofore unappreciated isoform of hCG—an early pregnancy associated molecular isoform of hCG.

[0073] hCG Urinary Analytes. Metabolites of hCG and hLH were studied in a variety of states (Birken, S., et al., 1996a). One study indicated a 31% pregnancy loss (Zina-

man, M J, et al., 1996) while another indicated a 17.4% rate of early pregnancy loss based on hCG assays (Ellish, N. J., et al., 1996). It is known that hCG and hCG beta core can be readily transferred from the uterus to the circulation even in the absence of implantation (Chang, P. L., 1997). The molecular spectrum of hCG urinary analytes in EPL cycles, normal conceptive cycles and non-conceptive cycles has been evaluated. The study design and demographics of the investigation have been described (Ellish, N. J., et al., 1996).

[0074] Briefly, three urine specimens per cycle, corresponding to days 9, 10, 11, post calculated day of ovulation were collected and analyzed in a screening assay (the "combo") which simultaneously detects intact, non-nicked hCG, hCG free beta subunit, and hCG beta core fragment. Individual determinations for each of these analytes, as well as for nicked hCG, and the form of intact hCG detected by monoclonal antibody B152 (EPMI hCG) were performed on these specimens. In addition, since the concentration of luteal phase hLH urinary analytes is a concern because of cross-reaction in hCG assays, levels of intact hLH, hLH free beta subunit and hLH beta core fragment were determined in the normal pregnancy cycles and the non-conceptive cycles. Table I summarizes the characteristics of immunometric assays employed.

TABLE I

Assay format and specificity		
Assay format	Primary analyte	% cross-reactivity
B109-B108*	intact non-nicked hCG	0%
B201-C104*	hCG free beta (non-nicked + nicked)	10% nicked hCG
B210-B108*	hCG beta core fragment	2% hLH beta core fragment
B151-B207*	hCG nicked	23% hCG nicked free beta; 14% hCG non-nicked; 14% hCG free beta; 14% hLH; 8% hLH free beta
B151-B604*	hCG nicked	3.7% hCG nicked free beta; 2.5% hCG non-nicked; <0.01% hCG free beta; <0.01% hLH; <0.01% hLH free beta
B152-B207*	hCG non-nicked and nicked (pregnancy and C5), hCG free beta nicked and non-nicked (pregnancy and C5), hLH, hLH free beta	31% hCG nicked (C5); 100% hCG free beta nicked (from C5); 25% hCG nicked (pregnancy); 79% hCG nicked free beta (pregnancy); 50% hCG (pregnancy); <1% hCG free alpha; <1% hCG beta core fragment; 27% hLH; 18% hLH free beta; 3% hLH beta core fragment
KB12-A201	hLH	0% hCG
B505-B503*	hLH beta core fragment	0% hCG
KB21-KB31*	hLH free beta	29% hLH intact

[0075] The results indicate that nicked hCG does not constitute a significant mole fraction of urinary hCG immunoreactivity in either EPL or early normal pregnancy. In addition, there is a substantial excretion of hCG free beta subunit in some subjects in both pregnancy and EPL. Further, both EPL and normal pregnancy cycles variably express all of the measured analytes. Although both the

incidence and level of expression are different between EPL's and normal pregnancy, there is no hCG related analyte unique to either state. There was, however, a clear difference between the hLH associated analytes in the control population (non-conceptive cycles) and the normal pregnancy group. Virtually all of the non-pregnancy cycles expressed hLH free beta subunit and hLH beta core fragment while only a third of the conceptive cycles had detectable levels of either analyte. Intact hLH proved to be a minor constituent of the hLH profile in both groups.

[0076] These findings demonstrate both the necessity of measuring hCG beta core fragment in the detection of EPL, and also of making sure that the hCG beta core assay does not cross-react with beta core hLH, which is demonstrated to be present in that part of the luteal phase where EPL measurements are performed. The data is summarized in FIG. 2.

[0077] Statistical analysis was performed after transformation of analyte values to mole fractions so as to produce a more useful analysis due to the wide excursion of hCG analyte values among groups. The mole fraction data were evaluated by discriminant analysis and by a mixed effects model incorporating LMP (last menstrual period date). The discriminant analysis was performed both with and without "outliers" (defined as values greater than two standard deviation from the mean) removed. Both approaches produced similar results.

[0078] A quadratic discriminant analysis based on a cross-validation method in order to minimize bias correctly classified 91% of the normal pregnancy subjects and 80% of the EPL subjects.

[0079] The mixed effects analysis, testing for interactions between mole fraction of analyte and time since LMP found no significant time or group (EPL vs. normal) effects in the intact hCG assay. In the free beta subunit of hCG assay, there is a significant group effect but no time trend. In both the

[0080] Nicked hCG. Several reports concerning the concentrations of nicked hCG during pregnancy and other states (Hoermann, R., et al., 1994; Cole, L. A., et al., 1993) are based on immunoassays using subtractive manipulations; subtracting results of one assay from that of a second assay to calculate the analyte. Such methods yield erroneous results. Specifically, obtaining values for nicked hCG by subtracting the values from an assay which does not recognize nicked hCG from an assay indifferent to nicking is not valid. The problem is that this approach will not work unless the epitopes detected in both assays are identical. This is apparent from the following data. For example, subtracting the hCG isoforms measured by the B109-B108*, standard non-nicked hCG assay, from the hCG concentrations measured by the new hCG assay which is oblivious to nicking, B152-B207*, does not yield the quantity of nicked hCG present but actually detects new isoforms of hCG not related to nicking of the polypeptide chain. Likewise, use of a scavenger antibody to remove interfering analytes can be effective but introduces inaccuracies in measurements as detailed below. In summary, a significant number of incorrect reports have been placed into the literature purporting to measure nicked hCG during pregnancy while little or none of this material is found during pregnancy using the specific assay systems developed and described herein.

[0081] Antibody production. The immunogen for the production of antibodies to nicked hCG was purified from a pool of normal pregnancy urine by a methodology previously described (Birken, S., et al., 1993). Following a standard procedure for hybridoma production (O'Connor J. F., et al., 1994), a total of seven monoclonal antibodies to normal pregnancy nicked hCG, and B151, a nicked hCG specific antibody derived using a choriocarcinoma derived 100% nicked hCG as immunogen, were produced and characterized. B152 (not shown in Table II) raised against the same immunogen as B151, recognizes a novel isoform of hCG associated with early pregnancy—designated herein, EPMI.

TABLE II

Characteristics of monoclonal antibodies								
Antibody	B601	B603	B604	B605	B606	B607	B610	B151
K_a, M^{-1}	5.3×10^8	8.9×10^6	1.2×10^9	1.4×10^9	$^*3.1 \times 10^8$	1.4×10^7	4.6×10^7	8.0×10^8
Isotype	IgG1,κ	IgG1, κ	IgG1, κ	IgG1, k	IgG1, κ	IgG1, κ	IgG1, κ	IgG1, κ

*to nicked hCGβ

hCG beta core fragment measurement and the B152 measurement, both the hormone levels and the time trend from LMP were significantly different between the EPL and pregnancy groups. This study produced several important findings. It defined the spectrum of analytes which in both early pregnancy and EPL, thereby resolving the issue of which hCG analytes to measure in epidemiological studies in which EPL is the end point determination. More importantly, it illustrated for the first time that there are significant differences both in the pattern of analytes and the time course of their appearance between early normal pregnancy and EPL. This observation facilitates very early prediction of a distressed pregnancy by urinary hCG measurements at a time which would permit therapeutic intervention.

[0082] Table II presents the binding constants and isotypes and FIG. 3 illustrates the specificity of these antibodies towards various hCG and hLH related analytes. It was found that in general, although it is possible to develop monoclonal antibodies with a primary specificity towards normal pregnancy nicked hCG, these antibodies tended to also cross react with hLH. It appears that nicking of the hCG molecule alters its conformation in such a way that epitopes common to both hLH and hCG are exposed.

[0083] After evaluation of all the combinations of nicked hCG monoclonal antibodies, the assay configuration which displayed the best sensitivity and specificity characteristics proved to be B151, a nicked specific monoclonal antibody derived from the C-5 (choriocarcinoma hCG) immunization as the capture antibody and B604, a monoclonal antibody

derived from immunization with the nicked hCG fraction purified from normal pregnancy urine, as detection antibody. The characteristics of the immunometric assay employing these two monoclonal antibodies are detailed in Table I (B15-B604*). This assay configuration provided a 2.5% cross-reactivity with intact, non-nicked hCG and 3.7% cross reactivity with nicked free beta subunit.

[0084] Table III details findings with respect to the content of urinary nicked hCG in normal pregnancy, ectopic pregnancy and spontaneous abortion, presented as a function of gestational age. The concentrations of non-nicked hCG are presented in the range of pmol/mg creatinine and those for nicked hCG are in fmol/mg creatinine. The assay for the nicked hCG has good but not absolute specificity (2.5% cross reaction with non-nicked hCG). Thus, nicked hCG does not constitute a substantial mole fraction (i.e. <5%-6%) of intact hCG immunoreactivity. A close correlation between blood and urine values for nicked hCG, indicating that the low level of nicked hCG found in urine is therefore not a consequence of preferential renal processing of circulating nicked hCG into smaller urinary molecular fragments. Coupling these observations with the very low incidence of expression of nicked hCG in the early normal pregnancy or EPL discussed previously, it is apparent that nicked hCG does not constitute a major constituent of urinary hCG in the first or second trimester of pregnancy.

[0087] These studies indicate that the quantities of nicked hCG are too low when compared with those of non-nicked hCG, to be of physiological significance. Furthermore, measurement of these small quantities in the presence of large excess of the non-nicked hCG isoform, even with a specific assay, are not accurate, due to intact hCG interference in the nicked hCG assay.

[0088] hCG-like immunoreactivity in periovulatory urine. Most of the "hCG like" immunoreactivity in urine was determined to be associated with hLH beta core fragment (hLHβcf). An assay (Kovalevskaya, G., et al., 1995) was developed which was capable of discriminating between the highly homologous hLHβcf and hCG beta core fragment (hCGβcf). This has permitted for the first time the dissection of the hLH/hCG immunoreactivity signal around ovulation. It was found that the predominant urinary molecular form in the periovulatory period was hLHβcf, with concentrations which are virtually an order of magnitude higher than either intact hLH or hLH free beta subunit (hLHβ). The contribution of hCG analytes, as judged by hCGβcf measurement is small in comparison to hLHβcf (O'Connor, J., et al., 1997).

[0089] Stability of Nicked hCG in Blood and Urine. Three types of stability experiments were performed. The stability of nicked hCG itself, purified from pregnancy urine was evaluated in buffer. The changes in measured endogenous nicked hCG in urine and serum pregnancy pools was also

TABLE III

Urinary Nicked and Non-nicked hCG in Pregnancy

Diagnosis	Age) (weeks)	n	(+) nhCG assay	nicked hCG fmol/mg crt		Non-nicked hCG* pmol/mg crt	
				median	range	median	range
normal	1.7–4.0	42	15	23	3–222	2.7	0.2–88.5
pregnancy	5–6	9	3	140	111–842	6.1	1.6–871.8
	10.6–14.9	17	17	971	36–6065	214.0	64.8–7184.5
	15–21.7	81	81	113	6–4718	60.5	7.3–2663.5
	28.1–39	6	6	46	28–93	30.2	16.5–138.1
ectopic pregnancy	2.3–4.3	9	4	6	3–20	3.6	1.2–6.7
spontaneous abortion	1.9–4.1	12	3	4.5	4–11	0.8	0.2–2.3

*Note that the concentration of non-nicked hCG is expressed in pmol/mg crt and nicked hCG in fmol/mg crt,
(+) = positive,
n = number of samples analyzed,
crt = creatinine

[0085] In order to be certain that some matrix effect was not interfering with detection of nicked hCG, its recovery from spiked hCG free serum and urine was evaluated. Recovery ranged from 69%-83% in serum and around 72% in urine. It became evident from recovery studies of nicked hCG in spiked pregnancy serum that the recovery determination was a function of the concentration of intact hCG in the specimen. (See FIG. 4A)

[0086] Although a scavenger antibody does function to remove a substantial part of the interfering analyte (hCG), which is usually present in large excess (as in pregnancy), it also alters the observed recovery of nicked hCG, due presumably to some cross-reactivity of the interfering hCG in the assay (FIG. 4B, FIGS. 5A and 5B).

evaluated because of possible "nicking enzymes" in biological fluids reported by other investigators (Kardana, A., 1996).

[0090] Sera and urine of pregnant women at different gestational ages were pooled and sodium azide (0.1%) was added. Urine pH was adjusted to pH 7 with 1M tris HCl. Aliquots stored at –80° C. served as control samples. Other aliquots were incubated at 4° C., 20 C. and 37° C. for extended time periods. After each time period the samples were stored at –80 C. All of the specimens were analyzed in the same assay. Nicked hCG in buffer (PBS/bovine IgG, sodium azide) which was treated with the same incubations and was analyzed in B151-B604* and B201-C104* assays.

[0091] With respect to stability in the buffer, two effects were noted. At 37° C., there was a rise in hCG free beta

subunit (hCGβ) due to the anticipated heat related increase in dissociation of the intact hCG molecule. Additionally, there was a decrease in nicked hCG over time after 20 hours of incubation, regardless of storage temperature, this occured even when there was no dissociation, as evidenced by hCGβ analysis. The effect was more pronounced at 20° C. and 37° C. than at room temperature. During a 70 hour time period the concentration decreased to about 50% of the initial value at 20° C. and 37° C. and to about 60% at 4° C. Although at elevated temperature, dissociation appears to be the predominant cause, at lower temperatures this is not the case, and the loss of nicked hCG immunoreactivity must be ascribed to destruction of the B151 epitope.

[0092] Endogenous pregnancy specimens of serum and urine stability determinations are confounded by the presence of a great excess of intact, non-nicked hCG, from which the nicked variant presumably arises. In both serum and urine, the apparent concentrations of nicked hCG remain relatively constant at 4° C., but increases with increasing time of exposure to elevated temperature. It appears from these data that the net rate of formation of nicked hCG (or increase exposure of nicked epitopes) as a result of heating is somewhat greater than the rate of destruction of nicked hCG resulting in an apparent increase in nicked hCG levels over time.

EXAMPLE 2

B152/B109 Ratio Predicts Pregnancy Outcome

[0093] B152/B109 immunoreactivity ratio as a function of gestational age. A total of 159 urine samples from normal pregnancies of known gestational age were assayed for intact hCG in the B109-B108* and B152-B207* assay configurations (See Table I). Over the gestational age range from 1-2 weeks to 40 weeks, the ratio of hCG isoforms as measured by the two assays decreased by nearly two orders of magnitude, with the greatest change occurring in the first fifteen weeks of gestation. See FIGS. 6-11. **FIG. 6** presents the second order regression curve (r=0.92) and 95% confidence intervals of the B152/B109 hCG isoforms ratio in urine as a function of gestational age (see also **FIG. 11**).

[0094] Analysis of matched blood and urine specimens in the first 5-6 weeks of pregnancy (n=10) and specimens obtained in the late third trimester, just prior to caesarian section (n=5) displayed a similar B152/B109 ratio inversion in serum as was observed in the urine from the same subjects. This data strongly suggests that the same change in the distribution of hCG isoform prevails in serum as observed in urine throughout the pregnancy (**FIG. 7**). It is of interest to note that the ratio of the two hCG forms in the JAR choriocarcinoma cell line, with a B152/B109 ratio of approximately two, supports the hypothesis that the form of hCG produced in early normal pregnancy differs in carbohydrate structure from that produced later in pregnancy and in fact may more closely resemble the hCG produced in trophoblastic malignancy which is characterized by a difference in the carbohydrate portion of the molecule as compared to the hCG derived from pregnancy. It has been reported that the hCG produced by choriocarcinoma has a higher biological activity than does the hCG from normal pregnancy (Wide, L., and Hobson, B., 1987). Studies indicate that early pregnancy isoforms of hCG have different biological activities when pregnancies are lost as compared

to pregnancies that continue (Ellish, N. J., et al., 1996). Thus, the B152-based is a sensitive immunoassay to detect critical isoforms of hCG.

[0095] Chromatography of First Week of Gestation Pregnancy Pool. In order to determine whether the B152-B207* assay recognized other forms of hCG associated immunoreactivity in addition to the intact hCG molecule, specimens were pooled. FPLC on tandem Superose 12 columns followed by immunoassay of the fractions for all of the characterized forms of hCG revealed that only the intact hCG molecule (or hCG free beta subunit) gave a signal in this assay (See **FIG. 8**). There were no lower molecular weight fragments identified by the B152-B207* assay. The hCG free beta analyte was measured in the 159 urines described in **FIG. 6** and was found to make a negligible contribution to over all hCG immunoreactivity in these specimens.

[0096] Molecules recognized by monoclonal antibody B152 in urine and pituitary extracts. In order to define the nature of the hCG isoforms recognized by B152, high resolution gel filtration columns of both pituitary extracts and postmenopausal urine concentrates were used (See **FIG. 8**). The rationale for use of pituitary extracts is to determine cross-reactive molecules, specifically those which are glycosylated, which are plentiful in pituitary which contains the entire family of glycoprotein hormones, hLH, hTSH, and hFSH as well as free subunits and the pituitary form of hCG. Two peaks are detected in both of these cases. Only one peak was detected in similar studies of pregnancy urine concentrates as described earlier. In the pituitary, it is likely that the larger molecule is pituitary hCG (70K) while the smaller sized molecule is hLH. Since hLH is present at 100x or so as compared to pituitary hCG, the apparent similar concentration of immunoreactivity indicates that B152 has reduced cross-reactivity to hLH as compared to hCG. Likewise, both hCG and hLH occur in postmenopausal urine, again with much more hLH than hCG and the B152 pattern is similar to that of the pituitary extract. These results show that B152 is generally hCG specific except for cross reactivity to hLH (as shown by standard cross-reaction studies in Table I) and that its carbohydrate specificity is both to the protein portion as well as to the carbohydrate moeities of hCG (and to a lesser extent of hLH) since it does not react with the multitude of other glycoyslated proteins present in the pituitary nor with those in postmenopausal urine except for hCG or hLH-related molecules.

[0097] Profiles of B152 immunoreactivity throughout normal pregnancy; correlation with receptor binding and in vitro biological activity. Matched blood and urine specimens are, collected at approximately weekly intervals throughout the duration of gestation (**FIG. 7**). These specimens are used to evaluate the correlation of assay values with in vitro receptor binding capacity and biological activity and to establish the range of time over which the transition of isoforms occurs in normal pregnancy.

[0098] Serum and urine specimens were analyzed using two assays, B109-B108* and B152-B207*, which recognize the difference in molecular isoforms of hCG. See Table. I. The in vitro bioassay for hLH/hCG is described above. (See **FIG. 1**). Results are indicated in FIGS. 6-11. The immunometric assay employs 96-well microtiter plate technology. The coating antibody, at a concentration determined to

provide the most satisfactory combination of sensitivity and range, is applied to the microtiter wells (Immulon IV, Dynatech Laboratories) in carbonate buffer (0.2M, pH 9.5). The plates are incubated with the coating solution at 4° C., overnight, then aspirated, washed with washing solution (0.05% Tween, 0.15N NaCl), and blocked with a 1% solution of BSA (three hours at room temperature). The BSA solution is aspirated and the appropriate hCG standards (200 μL/well), in buffer B (PBS/0.1% bovine IgG/0.1% sodium azide), or in hCG free serum (Chemicon, Inc.), or hCG free urine, as appropriate to the specimen matrix, and specimens are added to the wells. The plates are sealed with plate sealers, and incubated overnight at 4° C. The controls, specimens, and standards are then aspirated, the plates washed 5 times with washing solution, and iodinated detection antibody in buffer B (200 uL/well, 100,000 cpm/well) added and incubated overnight at 4° C. The wells are again aspirated, washed 5 times with washing solution, separated and counted (Packard Cobra gamma counted). Values are interpolated from a smoothed spline transformation of the count data. This assay procedure, as well as assay validation has been previously reported (O'Connor, J. F., et al., 1988).

[0099] Creatinine analysis, when urine values are normalized to creatinine, is performed in a microtiter plate format following a modification of the Taussky procedure (Taussky, H. H., 1954).

[0100] EPMI hCG in women with a history of recurrent spontaneous abortion and ectopic pregnancy. This experiment extended the observations of the significant difference in production of the hCG isoform preferentially recognized by the B152 antibody between successful and failing pregnancies. The specimens for this investigation were provided from women who experienced recurrent spontaneous abortions and ectopic pregnancies. Results are indicated in FIG. 10.

[0101] EPMI hCG in women undergoing embryo transfer. IVF patients are a group in which the rate of pre-clinical loss is presumably significant; the rate of successful clinical pregnancy in the IVF Program at Columbia University is 30% when non-donor sperm is used and 50% when donor sperm is used. A study on the effect of exposure to electromagnetic fields on EPL incidence with enrolled 650 subjects had approximately 10% daily collectors, the remainder collected 2 urine samples/cycle (first 2 days of menses). Of 167 clinical pregnancies there were 25 clinical losses and 34 putative early pregnancy losses. Of the live births, 7 are daily collectors as are 3 of the clinical losses.

[0102] Subjects collect small (5 mL) aliquots of first morning void urine. Urine collection commences three days before embryo transfer (baseline) and continues until the subject is confirmed to be clinically pregnant or not. This determination usually occurs at days 9 and 11 following embryo transfer. Blood specimens are also obtained on these subjects. Matched blood and urine specimens are obtained at various gestational ages. Serial blood and urine specimens Control specimens are obtained from patients who maintain their pregnancy full term. In addition to the B109-B108* and B152-B207* assays for intact hCG, hCGβ and nicked hCG determinations are performed on the serum and urine and additionally the hCGβcf assay on the urine specimens.

[0103] Descriptive statistical and graphical methods are used to measures of serum and urine samples from normal

healthy pregnancies to identify the distributions a) between patient first trimester average B152 levels, B109 levels and B152/B109 ratio; b) between patient variability in time to B152/B109 ratio reaching 1.00; and c) between patient variability in time to B152/B109 ratio declining by ⅓rd from first trimester maximum levels. The variability in the timing of the crossover in the ratio of these two analytes provides an empirical basis from which to estimate the value of these markers as biochemical signatures of a viable third trimester fetus.

[0104] Comparison of the assay profile of healthy normal pregnancies to those of unsuccessful pregnancies from failed IVF implantations, two non-parametric hypotheses are available: 1) the proportion of pregnancies in which the B152/B109 ratio falls below 1.00 is no different in healthy normal and unsuccessful IVF pregnancies; 2) the proportion of pregnancies in which the B152/B109 ratio declines by ⅓rd from first trimester maximum levels is no different in healthy normal and unsuccessful IVF pregnancies. These hypotheses can be tested as a difference between two proportions. For example, a comparison of week 14 vs. week 9, week 13 vs. week 6, week 12 vs. week 5 or week 11 vs. week 4 pregnancies to show a reversal of the B152/B109 ratio in healthy normal pregnancies and unsuccessful IVF implantations, respectively. The power analyses apply to an outcome defined as the time at which the B152/B109 ratio declines by ⅓rd from first trimester maximum levels, although this outcome would necessarily provide earlier detection of pregnancy failure than the reversal of the B152/B109 ratio. Patterns of results less discriminantly different from these indicate a rejection of the dichotomous outcome of B152/B109 ratio reversal as a clinically meaningful marker of pregnancy failure.

[0105] Alternatively, the same two non-parametric hypotheses can be recast as parametric hypotheses by considering the timing of the biochemical events within the assay profile of healthy normal pregnancies and unsuccessful pregnancies from failed IVF implantations: 1) the time at which the B152/B109 ratio falls below 1.00 is no different in healthy normal and unsuccessful IVF pregnancies; 2) the time at which the B152/B109 ratio declines by ⅓rd from first trimester maximum levels is no different in healthy normal and unsuccessful IVF pregnancies. Of course, the objective is to provide an empirical basis from which clinicians may counsel their patients. Thus, it is important to adopt a logistic model for this component of the data analysis. With pregnancy success as the outcome, logistic models allow the estimation of the (symmetrical) hypothesis of increase in risk of pregnancy failure for each additional week where either the B152/B109 ratio has failed to decline by one third from first trimester baseline maximum values or the B152/B109 ratio has failed to become less than 1.00 (measured in weeks). The logistic model enables specification of the time at which results indicate a particular pregnancy exceeds an a priori defined likelihood of failure, given assay data regularly available during pregnancy, and allows incorporation of other risks for pregnancy failure in the same data analytic framework to assess the relative contribution of threats to pregnancy loss. The Cox proportional hazard model may be used to examine predictors of the crossover rates. Mixed effects models can also analyze repeated measures of the B152/B109 ratios taken during entire cycles. These models are particularly useful since they allow inclusion of incomplete and imbalance data (i.e. data with miss-

ing values and unequal timing of data collection), to estimate effects of time-varying covariates, to model dependency structure of repeated measures and to model possible heterogeneity of the ratio measures within each experimental group.

[0106] B152 hCG isoforms isolated from early pregnancy urine and determination of their protein and carbohydrate structures. Using the already developed scheme of concentration and immunoaffinity extraction of urine, hCG molecules are isolated from urine collected from women in early pregnancy for both protein and carbohydrate analyses. According to one approach, molecules are isolated from HPLC fractions, digested with proteases before and after reduction of disulfide bonds, examination of the resultant peptides by mass spectrometry and/or sequence analysis, isolation of carbohydrate moieties after glycosidase digestions and determination of carbohydrate structures by a combination of specific glycosidases and retention times on specialized anion exchange columns as compared to know branch-chain oligosaccharide standards. In a similar approach, the final purification stage for the isolated hCG isoforms is SDS gel electrophoresis. Both protease digests and glycosidase digests are performed on the blotted and cutout band. This method results in greater purity of the protein and less artifactual errors due to contamination by carbohydrates which are not in the purified protein but are derived from outside contaminants.

[0107] Carbohydrate compositional analyses and oligosaccharide branched chain identifications. The MALDI TOF mass spectrometric method may be used to confirm oligosaccharide structures by using specific glycosidases on the glycopeptides and determining the change in molecular weight as the sugars are digested off the glycopeptide. Only the hCG beta COOH peptide can be expected to contain O-linked sugar moieties. These are of special interest since it is thought that B152 has significant reaction with this region. The structures of this region can be determined in a similar fashion using enzymes that specifically release O-linked glycans. The O-linked structures has been previously examined using standard reference pregnancy hCG (Cole, L. A., et al., 1985). The O-linked branched chain structure are determined by a similar strategy using the Dionex chromatographic system as well as specific glycosidases on the C-terminal glycopeptides and Mass Spectrometry. In one study (Elliott, M. M., et al., 1997), these techniques were used to elucidate the carbohydrate structures of CR series hCG preparations (standard urinary pregnancy hCG) and compared them to the structures of patient samples such as C5 which was the immunogen employed to generate antibody B152. It was found that C5 contained significantly more mono and tri-antennary (2×mono and 3×tri-structures than the CR preparations) on the N-Asn residues. It was also found that more tetrasaccharide structures were on the hCG COOH-terminal peptide O-Serine residues in the choriocarcinoma hCG isoform than in the CR preparations.

[0108] Biological activity and metabolic clearance of hCG isoforms. Biological activity is a function both of molecular structure and half-life in the circulation, which can be influenced by structure. Alterations in carbohydrate/sialic acid content of the glycoprotein hormones are thought to be responsible for the changes in hCG biological/immunological activity observed throughout pregnancy. In addition,

signal transduction at the receptor is influenced by the pI of the hCG isoform and the presence or absence of carbohydrate. Thus, it is valuable to examine both receptor binding and biological activity in vitro and, in order to determine the mechanism of action, to distinguish receptor binding and signal transduction as well as relative potency of signal transduction along with in vivo bioactivity determinants such as circulating half life. Studies, including clearance rates, are performed on B152 hCG isoforms of early successful pregnancy, hCG from third trimester pregnancy, and the reference urinary hCG preparation, CR 127.

EXAMPLE 3

B152 and B151 Immunoreactivity in
Non-Trophoblastic Malignancy

[0109] With the exception of trophoblastic disease and testicular cancer, hCG is expressed in the blood of about 20% of patients with all other types of cancer (Hussa, R. O., 1987). HCG beta core fragment in the urine has a significantly higher level of expression, especially in gynecological malignancy. Since the B152 antibody was developed to a form of hCG produced in a malignancy, it was of interest to examine the expression of B152 and nicked hCG immunoreactivity (B151) in non-trophoblastic malignancy. Accordingly, blood and urine derived from men undergoing chemotherapy for prostate cancer or women for ovarian cancer were evaluated for the expression of hCG isoforms in plasma and urine. It is significant that in prostate cancer, B152 hCG immunoreactivity is found in the blood and urine of prostate cancer patients in instances when there is no hCG detected by B109-B108*. In ovarian cancer patients evaluated, there is evidence of nicked hCG in the blood, even in the absence of both B109 and B152 immunoreactivity. Neither of the above groups demonstrated the presence of hCG immunoreactivity when the standard pregnancy derived hCG assay was employed. It is reassuring to find that nicked hCG, the existence of which has been documented by several investigators, can be found and reliably measured in a clinical setting.

[0110] Experimental Discussion

[0111] While it has been found that nicked hCG does not play a significant role in early pregnancy loss, a novel observation was made of previously unknown isoforms of hCG produced during very early pregnancy. In the course of these studies, a potentially important new signal was observed in the urine of women early in pregnancy, namely an epitope of a form of hCG which may indicate the likely success of carrying a pregnancy. Likewise, absence of this signal may indicate that EPL will occur. Since EPL can be a very sensitive marker of environmental toxins (Hakim, R. B., et al., 1995) and is frequently used as an epidemiological marker of exposure, the finding of this epitope provides a powerful tool for monitoring the safety of the environment. In addition, this assay facilitates increasing the success rate of IVF infertility programs since the predictive value of the new measuring system would rapidly indicate successful approaches. Described herein is the novel and completely unexpected finding that successful pregnancies display a high content of unique isoforms of hCG that are maintained for the first few weeks of pregnancy and then rapidly decline as pregnancy progresses. Based on properties of the immunoassay system, it is hypothesized that these hCG isoforms

may be hyperglycosylated. This is a striking observation never reported nor suspected earlier. Carbohydrate analyses (Elliot, M., 1997) demonstrate that Cs hCG employed as immunogen for antibody B152, contains two times the monoantennary content and three times the tri-antennary content of branch chain sugars as compared to the CR series of natural pregnancy urinary hCG. In addition, the O-linked carbohydrates are mostly tetrasaccharide instead of disaccharide in CS as compared to CR 127 hCG. (CR 127 hCG is similar to the WHO preparation, the third international hCG standard, which was CR 119 hCG, prepared by Canfield and Birken twenty years ago but still in use today-)(Birken, S., et al., 1991a). B152 recognizes CS hCG much better than nicked CR127 hCG or non-nicked CR 127 hCG (Birken, S., et al., 1993). In addition, JAR cell type hCG is known to contain a similar array of carbohydrate moieties. It was found to be recognized by B152 similar to the early pregnancy isoforms in healthy pregnancies. The observation that the hCG isoform produced by JAR cells in culture (B152/B109 ratio) is similar to that found in early pregnancy hCG isoforms supports the hypothesis that the production of a type of hCG with a particular glycosylation pattern is a prerequisite for a viable pregnancy. This glycosylation pattern is not characteristic of the hCG of later pregnancy.

[0112] A variety of pregnancy disorders are testable. One category of patients consists of those women who experience a high rate of recurrent abortions. Even in populations with no known fertility problems, the total rate of pregnancy loss is 32% (EPL plus clinically recognized abortion) (Wilcox, A. J., et al., 1988). The risk of recurrent abortion increases with the number of spontaneous abortions experienced in the past, reaching an incidence of 32% after three consecutive abortions. (Hill, J. A., and Anderson, D. J., 1990). Probable causes of recurrent spontaneous abortion, comprising genetic, infectious, hormonal imbalance, or immunologic factors can be established in less than 60% of all spontaneous abortions, leaving 40+% of spontaneous abortions with a completely unestablished etiology. These facts, taken together with reports establishing that the administration of exogenous hCG can be an effective therapy in subjects with a history of recurrent spontaneous abortion (Quenby, S., and Farquharson, R. G., 1994; Harrison, R. F., 1985) lends support to the hypothesis that a disproportionate production of the ineffective isoforms of hCG in early pregnancy is a causal factor in both early pre-clinical loss as well as in spontaneous abortion.

[0113] A second category includes women undergoing embryo transfer. These patients provide several distinct advantages: The patients undergoing this procedure are not treated with crude hCG preparations, making measurement of hCG isoforms easy and decisive since all hCG forms derive from the embryo none from any injected hCG preparations. Second, is the opportunity to monitor the nature of the isoforms from day 9 of a successful pregnancy. Third, is the ability to obtain large volumes of urine to purify the early pregnancy isoforms to determine their structures. Fourth, since pregnancy loss is from 50% to 70% in this population, the loss can be defined as due to lack of the essential hCG isoform recognized by B152 or due to other causes. Comparison of early pregnancies in populations of women not undergoing in vitro fertilization procedures with those undergoing embryo implantation can, thus, assess whether pregnancy loss situations present similar or different patterns of hCG isoforms during the process. The mechanism of

pregnancy loss in the general population as compared with the much higher rate of embryo loss in IVF programs may be different. Additionally, it has been established that the hCG produced in choriocarcinoma has differences in carbohydrate structures, sialic acid content and biological activity (Wide, L., and Hobson, B., 1987; Elliot, M., et al., 1997; Hussa, R. A., 1987). Since both the B151-B604* and B152-B207* assays incorporate monoclonal antibodies raised against an immunogen derived from choriocarcinoma, specimens may be evaluated from patients with gestational trophoblastic disease in order to determine whether the above assays recognize the hCG produced in these conditions with greater sensitivity and specificity than do assays based on the hCG of normal pregnancy, as is apparently the case for the hCG produced in testicular and ovarian cancer.

[0114] There are few reports of changes of carbohydrate content of hCG-related molecules during pregnancy. Blithe and colleagues studied free alpha subunit of hCG whose carbohydrate content differs from that of alpha within hCG by additional carbohydrate antennae and fucose. The carbohydrate of free alpha becomes increasingly complex in terms of more branches and higher content as pregnancy proceeds. It has also been reported that the quantity of fucose increased in both hCG and in free alpha as pregnancy proceeded (Skarulis, M. C., et al., 1992). Thus, the literature indicates increasing content and complexity of carbohydrate of hCG and free alpha subunits. However, immunological data using the B152 monoclonal antibody, implies a progression to simpler carbohydrate content during pregnancy. Since the beta COOH-region's O-linked carbohydrates may be involved in the epitope recognized by B152, it is conceivable that the carbohydrate structures of this region may be altered in a different pattern from the N-linked glycans studied by Blithe and colleagues (Skarulis, M. C., et al., 1992; Blithe, D. L., and Iles, R. K., 1995). Data from Skarulis et al. indicate that heterodimeric hCG may contain additional fucose but do not provide data that this late pregnancy hCG becomes hyperglycosylated as does free alpha.

[0115] Other studies indicated that the forms of hCG during EPL likely differ in biological activity from those hCG isoforms in successful pregnancies (Ho, H.-H., et al., 1997). The in vitro bioassays employed in those studies are unsuitable for large-scale studies and are not as reliable as the immunoassays described herein. Furthermore, it is likely that in vivo assays may give different results since in vitro and in vivo assays sometimes give completely disparate results. In this case, in vivo and clearance assays are most important in order to identify whether the hCG isoforms are truly more potent in the whole animal and to identify the reasons for the increased potency. Thus in vitro and in vivo bioactivities of the early pregnancy isoforms of hCG are highly significant.

[0116] Carbohydrate differences is a widely accepted explanation for variations in biological to immunological ratio such as the forms observed by various studies of EPL (Ho, H.-H., et al., 1997). Various studies (Grotjan, H. R. J., and Cole, L. A., 1989; Hoermann, R., 1997; Stanton, P. G., et al., 1993; Szkudlinski, M. W., et al., 1995, Thotakura, N. R., et al., 1994; Szkudlinski, M. W., et al., 1993), have shown that sialic acid differences are an explanation for such heterogeneity in biological activities of glycoprotein hormones. These studies have also confirmed the dogma that in

vitro biological activities can yield the opposite results from in vivo studies because of altered metabolic clearance rates in the latter studies. Thus, more acidic (more highly sialylated) forms of gonadotropins are more biopotent in the whole animal because of prolonged circulating half-lives. The same molecules may appear less potent in in vitro assays due to greater acidity, greater negative sialic acid content. Hoermann et al. (Hoermann, R., et al., 1997) demonstrated the exclusion of many of the acidic circulating hormone forms from the urine, thus, prolonging their half-lives. The pI pattern of normal pregnancy as well as trophoblastic cancer hCG in serum is quite different from that of urine. Since the studies described herein indicate that EPL hCG isoforms have reduced in vitro biological activity, this finding cannot be explained solely by what is known of biological activity and sialic acid content. Early pregnancy isoforms recognized by monoclonal antibody B152 may be more potent in vivo by virtue of prolonged half-life they may then display increased signal transduction at the receptor as well. This may be explained by a hyperglycosylated form of hCG which is not hypersiaylated. In this case, the extra sugar portion would help prolong circulating half-life of a more basic pI form of hCG which also has increased in vitro bioactivity.

EXAMPLE 4

Diagnosis of Gestational Trophobloast Disease

[0117] An important application of the B152 (early hCG isoform)/B109 (late hCG isoform) ratio analysis described herein above is in the very early (and facile) diagnosis of gestational trophoblast disease. Examples of gestational trophopblast disease include choriocarcinoma or hydatidiform mole. In normal pregnancy, the ratio of B152/B109 of the two isoforms of hCG rapidly decreases, eventually inverting. In gestational trophoblast disease including choriocarcinoma or hydatidiform mole, the ratio is initially higher than found in normal pregnancy, but does not diminish during the course of the apparent pregnancy. This approach provides a highly sensitive and specific diagnostic marker for gestational trophoblast disease.

[0118] Other pregnancy disorders in which hCG levels are abnormally high or abnormally low include Down's syndrome or other aneuploid pregnancies, ectopic pregnancy, preeclampsia, and intra-uterine growth retardation. Because the hCG production in these conditions is quantitatively abnormal compared with normal pregnancy, an altered ratio of the hCG isoforms identified by B152 (early hCG isoform) and B109 (late hCG isoform) can be detected.

[0119] Thus, the dual isoform analysis (B152/B109) further provides a method for diagnosing pregnancy disorders and gestational trophoblast disease.

[0120] Experimental Procedures

[0121] Hormones. hCG CR127 was prepared and characterized as described earlier (O'Connor et al. 1994, Birken et al. 1991). Nick-free hCG (preparation 814) was separated from parent CR 127 preparation by hydrophobic chromatography. Procedure was a modification of the separation of Phenyl Sepharose described earlier (Birken et al. 1993). CR 127 hCG (26 mg) was dissolved in 0.6M ammonium sulfate buffer containing 0.05M ammonium bicarbonate. This solution (3 ml) was loaded onto a Pharmacia Hi Load Phenyl

Sepharose Pre-packed column and eluted by a wash of 90 ml of starting buffer followed by a batch elution with 210 ml of 0.05M ammonium bicarbonate. During this step of the separation, nicked hCG eluted along with a small quantity of non-nicked hCG of presumably a more hydrophilic form of non-nicked hCG than are the majority of non-nicked hCG molecules. Next, the major component of non-nicked hCG was eluted by applying 40% ethanol in 0.05M ammonium bicarbonate (90 ml).

[0122] Nicked hCG (preparation 813) was purified from parent CR 127 preparation by hydrophobic chromatography as described for non-nicked hCG.

[0123] Nicked hCG beta (preparation 834) was separated from CR 129 hCG beta by reverse phase chromatography in 0.1% TFA/acetonitrile buffer (Birken et al. 1991).

[0124] HCG beta core fragment (preparation 455) was prepared from Diosynth crude commercial hCG by modification of the method of Blithe (Blithe et al. 1988) gel filtration, Concanavalin A chromatography, and anion exchange, followed by reverse phase chromatography in 0.1% TFA/acetonitrile.

[0125] Other hormones: hLH (AFP-8270B), hLHβ (AFP-382) were kindly provided by the National Hormone and Pituitary Program, NIDDKD. HLHβcf were prepared as described by Birken (Birken et al. 1993).

[0126] Purification of Monoclonal Antibodies. Immunoglobulins were purified from ascites by the Protein A Monoclonal Antibody Purification System (Bio-Rad, Richmond, Calif.). The protein concentration of pure antibodies was determined by amino acid analysis. Purification of mABs was checked by a PAGE in the presence of SDS according to the method of Laemmli (Laemmli, 1953).

[0127] Iodination of Hormones And Monoclonal Antibodies. Antibodies and hormones were labeled with 125I by the chloramine T-method (Hunter and Greenwood, 1962).

[0128] Immunization of Mice And Cell Fusion. One group of Balb/c mice was immunized with nicked hCG (preparation 813), another was immunized with nicked hCGb (preparation 834) according the following protocol: the first immunization was carryed out subcutaneously with 15-20 μg of immunogen per animal in complete Freund's adjuvant; the second was carried but after two weeks with the same amount of intraperitoneally (ip) using 15 μg of antigen in PBS for each animal immune sera were tested for antibodies in liquid phase radioimmunoassay (RIA) using ^{125}I-nicked hCGb. Mice with a high immune response were boosted with 15 μg hormone and after 3 days used for fusion.

[0129] Splenocytes from immunized mice were fused with cells of myeloma line X63-Ag8,653 (American Type Culture Collection) 3 days after the booster injection according to the method of Kohler and Milstein (Kohler and Milstein, 1975) as described in (Kovalevskaya et al. 1995). The splenocyte to myeloma cell ratio was 4:1 or 6:1. Polyethylene glycol 4000 (Sigma, St. Louis, Mo.) was used as the fusing reagent. On days 12-14 post fusion, culture supernatants (100 μl) from the wells with cell clones were screened for the presence of antibodies to hLHβcf using a liquid phase RIA. Positive selected cells were cloned at least two times by limiting dilutions on mouse peritoneal feeder cells. Subclones were injected ip into Balb/c mice (0.5×10⁶ cells/ mouse and mAB were purified from the ascites.

[0130] Isotypes of mABs were determined using Immu-noPure Monoclonal Antibody Isotyping Kit II (AP/PNPP) (Pierce, Rockford, Ill.) according to the manufacturer's instruction for the antigen-dependent technique.

[0131] Screening of Primary Clones and Immune Serum. Primary screening was carried out in a liquid phase RIA with 125I-nicked hCGb. The liquid phase RIA procedure has been described earlier (Birken et al., 1980). Briefly, the binding buffer consisted of PBS supplemented with 0.1% BSA and 0.1% sodium azide. 150 μl solution containing 30,000-40,000 cpm ^{125}I-nicked hCGb was added to 100 Al culture supernatant diluted 25:1 with PBS. 50 μl of 8% normal mouse serum was also added. This solution was first incubated fro 1 hour at 37° C. and then overnight at 4° C. Following incubation, 500 μl of a 2.5% goat anti-mouse serum was added and mixture was incubated for 1 h at 37° C. followed by 2 hours at room temperature. The precipitate containing bound radioactive hormone was separated by centrifugation and counted in a gamma counter. Superna-tants of positive clones were tested in the same type of assay to check cross-reactivity with ^{125}I-hCG and ^{125}I-hCGb. Immune serum was used as a positive control.

[0132] Competitive Liquid Phase RIA. Competitive liquid phase radioimmunoassays have been described previously (Kovalevskaya et al. 1995). Briefly they were conducted as follows: Cell supernatants were used at those dilutions at which approximately 40% of maximum antibody binding occurred in the absence of unlabeled hormones. The follow-ing regents were added to each 12x75 mm polystyrene tube: 100 μl diluted supernatant, 30,000-40,000 cpm of ^{125}I-nicked hCG or ^{125}I-nicked hCGb in 300 μl binding buffer (PBS), pH 7.2 with 0.1% BSA), 100 μl competitor solution and 100 μl 8% normal mouse serum. After incubation for 1 hour at 37° C. and overnight at 4° C., 1 ml of 2.5% goat anti-mouse serum was added as in the primary screening. Affinity constants were calculated by homologous competi-tive displacement assays using the PC version of the pro-gram Ligand by Munson (Munson and Rodbard, 1980).

[0133] Immunometric Assay For Nicked HCG. The meth-odology for the construction and validation of immunomet-ric assays has been fully described (O'Connor et al., 1988). Briefly, the specificity of the antibody pairs and their capac-ity for simultaneous binding to antigen are determined as follows. The analytes tested for potential cross-reaction with the nicked hCG monoclonal antibodies included hCG, hCGβ, nhCGb, hCGβcf and hLH. The degree of cross reaction was anticipated from a knowledge of antibody specificity in liquid phase RIA.

[0134] The B151 antibody was absorbed onto the wells of microtiter plates (Immunlon IV, Dynatech, Chantilly, Va.) by incubating a 10 μg/ml solution in coating buffer (0.2 M bicarbonate, pH 9.5) overnight at 4° C. The coating antibody solution was aspirated, the plates washed (wash solution: 0.9% NaCl, 0.05% Tween 20) and blocked with a 1% solution of BSA in PBS with 0.1% sodium azide. Following incubation with the BSA solution (minimum 3 hours at room temperature) the blocking solution was removed, the wells again washed with wash solution and 200 μl/well of the appropriate nicked hCG standards or potential cross-react-ing molecules were added in phosphate buffer B (0.05M phosphate with 0.1% bovine gamma globulin and 0.1% sodium azide) or in hCG-free serum (for serum assay)

(Chemicon, TEMECULA Calif.) After overnight incubation 4° C., the plates were again aspirated and washed. The 200 μl (50,000 cpm-100,000 cpm) of B604 ^{125}I-labeled antibody was added to the wells which were again incubated for 24 hours at 4° C. The tracer was aspirated, the plates washed with wash solution, the individual wells placed in glass tubes and the radioactivity determined in a Packard Cobra gamma counter. Doses were determined by interpolation from a smoothed spline transformation of the data points.

[0135] In addition to the nicked hCG assay, assay B109-B108* for intact hCG was employed. Prior to assay, the urines were thawed, the pH is adjusted with 1.0M Tris (pH 9.0), 50 μl/ml urine. The assay is performed from that point identically to that described for antibody characterization.

[0136] Recovery of Nicked HCG. Nicked hCG was assayed as described above using B151-B604* assay in the presence of increasing concentration of hCG (1.76-176 pmol/ml) with or without 10 g/ml B109 as a scavenger for hCG.

[0137] Subjects. Trophoblast serum and samples, Down syndrome samples and control normal pregnancy urine samples, ectopic pregnancy and spontaneous abortion samples were kind gift of Dr. L. Cole (Yale University). Matched serum/urine samples (5-6 weeks gestation age) obtained from practice CPMC physicians.

[0138] Creatinine. Creatinine determinations were per-formed in a 96-well microtiter plate format (Taussky, 1954).

[0139] Urine Processing. Twenty-four hour urine samples are collected from women undergoing embryo transfer as well as women in early natural pregnancy. The urine is refrigerated during the collection procedure. After delivery of the urine to the laboratory, sodium azide is added to 1 g/liter. Women undergoing in vitro embryo transfer are not pre-treated with hCG. Thus, all hCG which appears in their blood or urine is derived from the embryo (except for the small amounts of pituitary hCG present in all people). Raw urine is freed from particles by centrifugation followed by Pellicon filtration through a 0.45 micron membrane. Next, the procedure is to concentrate the urine with a Pellicon (Millipore) system which concentrates as much as 30 liters to 500 ml overnight (4° C.) using a 3,000 MW cutoff membrane. Smaller volumes can be concentrated in less that two hours. Next, the urine is desalted and delipidated by passage through a large volume of Sephadex G25 in 0.1 M ammonium bicarbonate. This step greatly increases the binding of CG to immunoaffinity columns. The desalted urinary concentrate is next size fractionated on the Pharma-cia HiLoad Superdex 200 and the hCG and hCG subunit peaks are identified by specific immunoassays (O'Connor, J. F.; et al., 1994) and the appropriate fractions are pooled and dried. The hCG and hCG subunits are purified from the gel filtered urine concentrate by immunoaffinity on insolubi-lized hCG antibody columns as described but with the use of either 4M guanadine (0.1M tris acetate, pH 5) or ammonium thiocyanate as eluant to decrease loss of sialic acid from the hormone. Alternatively, hCG is purified by conventional chromatographic procedures, anion exchange and hydro-phobic chromatography. The subunits are separated on reverse phase HPLC using a 0.01M sodium phosphate, pH 5 buffer and acetonitrile, after incubation in 4M guanadine,

0.1M tris acetate, pH 5. A third method is final purification and separation of the hCG subunits on SDS PAGE electrophoresis followed by electroblotting to PVDF. The PVDF band can be subjected to protease digestion to release peptides and glycopeptides which can be separated on reverse phase HPLC in neutral pH 5 buffers.

[0140] Separation of Glycopeptides from Isolated hCG subunits. To facilitate isolation of the glycopeptides from the hCG subunits, the subunits are both tryptic digested and the products of digestion are separated on reverse phase HPLC (using a pH 5 buffer). This procedure results in removal of the large beta COOH-terminal peptide which contains O-linked sugars. It also releases small, non glycopeptides from both subunits (Pollak, S., et al., 1990, Birken, S., et al., 1987; Birken, S., et al., 1986). Next, the main disulfide-linked core of each hCG subunit, is reduced and carboxymethylated, and separated on reverse phase HPLC at pH 5. At this stage, large peptides are isolated, including the glycopeptides. Each separated glycopeptide is redigested with trypsin and re-separated on HPLC at pH 5. These glycopeptides are next employed for two different methods of sugar chain analysis. One method is the approach of releasing the oligosaccharides by enzymatic digestions uing PNGase F for the N-linked glycans. The released-glycans can be separated from the peptides by ethanol precipitation, desialyated with neuraminidase, and separated directly on a Dionex Carbopac PA-100 column. Oligosaccharide standards are available from Dionex, Oxford Glycosystems and other companies for calibrating column elution times for various glycans (Hardy, M. R., and Townsend, R. R., 1994, Rohrer, J. S., et al., 1993, Weitzhandler, M., et al., 1993; Townsend, R. R., et al., 1989). Confirmation of the released structures is obtained by performing carbohydrate compositional analysis of eluted glycan peaks as well as performing digestions with specific glycosidases and rechromatographing the modified glycan on the Dionex system (Hardy, M. R., and Townsend, R. R., 1994; Rohrer, J. S., et al 1993; Weitzhanlder, M., et al., 1993; Townsend, R. R., et al., 1989; Townsend, R. R., et al., 1991; Townsend, R. R., et al., 1989; Hardy, M. R., and Townsend, R. R., 1989; Townsend, R. R., et al., 1988; Hardy, M. R., et al, 1997; Hardy, M. R., and Townsend, R. R., 1988; Dionex, 1997; Spellman, M. W., 1990; Kumarasamy, R., 1990). The newly modified glycan can be observed to elute at the same time as the appropriate standard oligosaccaharide and, in addition, the released monosaccharide can frequently be identified as well (Dionex, 1997). Structure determination is facilitated by the use of specific glycosidases for branch chain cleavage as well as for digestion of individual sugars from each of the branch chains. For example, Endo H cleaves high mannose type and hybrid oligosaccharide chains while glycosidase Endo F2 cleaves biantennary complex types and PNAase F cleaves tri and tetra-antennary chains down to the N-Asn bond.

[0141] Competitive receptor binding and in vitro bioassay. Bioassays are performed with recombinant-engineered CHO cells transfected with the human receptor to LH/CG Cells are maintained in Ham's F-12 medium, 4 mM Glutamine, 400 ug/ml G418 (Gibco), 5% fetal calf serum, 100 IU/ml penicillin, 100 ug/ml streptomycin. The cells are removed from the flask surface by versene only.

[0142] A competitive receptor assay constructed as follows: The receptor binding assay mixture contains 100 ul of the appropriate dilution of serum/urine samples or hCG

dilutions for standard curve, 100 ul of ^{125}-I-hCG (50,000-100,000 cpm) in buffer A(PBS/0.1%BSA) and 100 ul of CHO cells (2×10^5 cells in PBS). The mixture is incubated at 37° C. with slight shaking followed by centrifugation for 10 minutes at 750×g. The supernatant is aspirated and the cell pellet is counted in gamma-counter.

[0143] In vitro bioassay. Transfected CHO cells are seeded (200,000 cells/well) into a 24 well plate in culture medium and incubated for 2-3 days until the cells reach confluence. Non-transfected CHO cells are included to monitor non-specific response. The medium is removed and replaced with medium containing 1 mM isobutylmethylxanthine with appropriate dilutions of tested serum or urine. The plates are incubated at 37° C. for two hours. The supernatant is removed, and the wells washed with Hank's balanced salt solution. The intracellular cAMP is extracted with 95% ethanol, which is diluted 1:5, (or up to 1:40, depending on cAMP content) in assay buffer provided by the cAMP kit (New England Nuclear). cAMP assay is performed according to manufacturer's instructions. Response is normalized to well protein content (BCA protein assay kit, Pierce, Rockford, Ill.).

[0144] In vivo bioassay is determined by the uterine weight assay in immature female mice, following the procedure of Wide and Hobson (Wide, L., and Hobson, B., 1987). The mice are injected subcutaneously with one third of the total dose of gonadotropin on three consecutive days and killed 72 hours after the first injection. Uteri are dissected free from mesentery, fat and oviducts, blotted to remove intrauterine fluid and weighed to the nearest 0.1 mg. Five to ten mice are used at each of these dose levels. The hCG standard preparation used is a nicked hCG. This material may be run concurrently with specimens isolated from first and third trimester pregnancy. Sham saline injection may be used as a control. The response signal is the log mouse uterine weight.

[0145] Clearance of hCG isoforms. The clearance of hCG is determined in the rat. Blood (200 ul/sample) is obtained at 0, 120, 240, 360 and 480 minutes post injection, from an indwelling catheter in an catheterized external jugular vein, following the procedure described by Newman et al. (Newman, C. B., et al., 1985) and Brown and Hedge (Brown, M. R., and Hedge, G. A., 1972). Briefly, adult male Sprague-Dawley rats (Charles River Laboratories, Wilmington Mass.), wt 175-225 g, are given free access to food and water. Rats are handled for acclimatization for one week after arrival, and several days before the hCG infusion, the rats are cannulated under pentobarbital anesthesia. A 21 gauge stainless steel cannula is inserted into the one external jugular vein. The placement of the catheter allows for the collection of blood from the unrestrained, unstressed rat. After the animals have recuperated from the cannula implacement, an hCG isoform is injected (10 μg/ml sterile saline) through the cannulated vein. Blood samples are obtained at the four time intervals listed above. The blood is allowed to clot and the serum separated and stored at −80° C. for immunometric assays specific for different hCG isoforms.

[0146] Clearance rate of the isoforms of hCG from the circulation of the rat are estimated by computer fitting the concentration data to an equation of the general form: Concentration=$Ae^{-\alpha t}$+$Be^{-\beta t}$ at time t; A and α are parameters

of the rapid component and B and β are parameters of the slow component. The metabolic clearance rate (MCR) is calculated as MCR=Dose/(A/α+B/β) and the initial volume of distribution is calculated from V_d=Dose/(A+B). The MCR is normalized to body weight for statistical analysis, which is performed using ANOVA with Duncan's range test for determination of significance (Cassals, J. W., et al., 1989).

[0147] Mice. The mouse species used in the experiments described herein are Balb/c mice, aged 12-20 weeks old and adult Sprague-Dawley rats of either sex. Mice used for the production of monoclonal antibodies through ascites and for the determination of in vivo biological activity as described. Balbc/c mice are used because hybridoma cell lines were developed using Balb/c splenocytes.

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What is claimed is:

1. A method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising:

(a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG;

(b) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and

(c) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein the relative absence of the early pregnancy associated molecular isoform of hCG in the sample indicates a negative outcome of pregnancy for the subject.

2. The method of claim 1, wherein the antibody is B152.

3. The method of claim 1, step (a) further comprising a second antibody which specifically binds to hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG.

4. The method of claim 3, wherein the second antibody is B207.

5. The method of claim 1, step (a) further comprising a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG.

6. The method according to claim 5, wherein the second antibody is B108 or B109.

7. The method of claim 5, step (c) comprising comparing the amount of the early pregnancy associated molecular isoform of hCG determined in step (b) for said antibody with the amount determined in step (b) for the second antibody, wherein a high ratio of amounts determined for said antibody relative to the second antibody indicates a positive outcome of pregnancy for the subject, a low ratio indicates a negative outcome of pregnancy for the subject.

8. The method of claim 1, step (c) comprising comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

9. The method of claim 1, wherein the sample is a urinary sample or a blood sample.

10. The method of claim 1, wherein the sample is an aggregate sample taken from at least two consecutive days.

11. The method of claim 1, wherein the antibody is labeled with a detectable marker.

12. The method of claim 11, wherein the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin.

13. The method of claim 12, wherein the radioactive isotope is I^{125}.

14. A method of predicting pregnancy outcome in a subject by determining the amount of an early pregnancy associated molecular isoform of hCG in a sample comprising:

(a) contacting a capturing antibody which specifically binds to the early pregnancy associated molecular isoform of hCG with a solid matrix under conditions permitting binding of the antibody with the solid matrix;

(b) contacting the bound matrix with the sample under conditions permitting binding of the antigen present in the sample with the capturing antibody;

(c) separating the bound matrix and the sample;

(d) contacting the separated bound matrix with a detecting antibody which specifically binds to hCG under conditions permitting binding of antibody and antigen in the sample;

(e) measuring the amount of bound antibody on the bound matrix, thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample;

(f) comparing the amount early pregnancy associated molecular isoform of hCG in the sample determined in step (e) with either (i) the amount determined for temporally matched, normal pregnant subject(s) or (ii) the amount determined for non-pregnant subject(s), wherein amounts of the early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in temporally matched pregnant samples indicates a positive outcome, amounts of early pregnancy associated molecular isoform of hCG in the sample similar to amounts of early pregnancy associated molecular isoform of hCG in the non-pregnant samples indicates a negative outcome of pregnancy for the subject.

15. The method of claim 14, further comprising:

(a) removing of the sample from the matrix; and

(b) washing the bound matrix with an appropriate buffer.

16. The method of claim 14, wherein the capturing antibody is B152.

17. The method of claim 14, wherein the detecting antibody is B207.

18. The method of claim 14, step (a) further comprising a second capturing antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG.

19. The method according to claim 18, wherein the second capturing antibody is B108 or B109.

20. The method of claim 14, step (d) further comprising a second detecting antibody which specifically binds to hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG.

21. The method of claim 14, step (f) comprising comparing the amount of the early pregnancy associated molecular isoform of hCG determined in step (e) for said antibody with the amount determined in step (b) for the second antibody, wherein a high ratio of amounts determined for said antibody relative to the second antibody indicates a positive outcome of pregnancy for the subject, a low ratio indicates a negative outcome of pregnancy for the subject.

22. The method of claim 14, wherein the sample is a urinary sample or a blood sample.

23. The method of claim 14, wherein the sample is an aggregate sample taken from at least two consecutive days.

24. The method of claim 14, wherein the antibody is labeled with a detectable marker.

25. The method of claim 24, wherein the detectable marker is a radioactive isotope, enzyme, dye, magnetic bead, or biotin.

26. The method of claim 25, wherein the radioactive isotope is I^{125}.

27. A method for determining the amount of early pregnancy associated molecular isoforms of in a sample comprising:

(a) contacting the sample with an antibody which specifically binds to an early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG; and

(b) determining the amount of complexes formed thereby determining the amount of early pregnancy associated molecular isoform of hCG in the sample.

28. The method of claim 27, wherein the antibody specifically binds a region of the early pregnancy associated molecular isoform of hCG comprising a carbohydrate moiety.

29. The method of claim 27, wherein the antibody is produced by the hybridoma cell line accorded ATCC Accession No. _____.

30. The method of claim 27, wherein the antibody is B152.

31. A diagnostic kit for determining the amount of early pregnancy associated hCG is a sample comprising:

(a) An antibody which specifically binds to an early pregnancy associated molecular isoform; and

(b) a solid matrix to which the antibody is bound; and

(c) reagents permitting the formation of a complex between the antibody and a sample.

32. The diagnostic kit of claim 31, wherein the antibody is B108, B109 or B152.

33. The diagnostic kit of claim 31, further comprising control sample(s) normal pregnant sample(s), nonpregnant sample(s), or male sample(s).

34. The diagnostic kit of claim 31, wherein the antibody is labeled with a detectable marker.

35. The diagnostic kit of claim 34, wherein the detectable marker is a radioactive isotope, enzyme, magnetic bead, dye or biotin.

36. The diagnostic kit of claim 35, wherein the radioactive isotope is I^{125}.

37. An antibody which specifically binds to an early pregnancy associated molecular isoform of human chorionic gonadotropin.

38. The antibody of claim 37, wherein the antibody specifically binds to a region of the early pregnancy associated molecular isoform of human chorionic gonadotropin comprising a carbohydrate moiety.

39. The monoclonal antibody of claim 37 designated B152.

40. A hybridoma cell accorded ATCC Accession No. _____, producing the monoclonal antibody of claim 39.

41. The early pregnancy associated isoform of hCG of claim 1.

42. The early pregnancy associated isoform of hCG recognized by the monoclonal antibody of claim 39.

43. A method for detecting non-trophoblast malignancy in a sample comprising:

(a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG;

(b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG.

(c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample; and

(d) comparing the amount of early pregnancy associated molecular isoform of hCG in the sample determined in step (b) with the amount of early pregnancy associated molecular isoform of hCG in the sample determined in step (c), wherein a positive detection of early pregnancy associated molecular isoform detected in step (b) and a relative absence of the early pregnancy associated molecular isoform of hCG detected in step (c) indicates the presence of non-trophoblast malignancy in the sample.

44. The method of claim 43, wherein the antibody is B604, B151, B152 or B207.

45. The method of claim 43, wherein the second antibody is B108, B109.

46. The method of claim 43, wherein the non-trophoblast malignancy is ovarian malignancy or prostate malignancy.

47. The method of claim 43, wherein the sample is a urinary sample or a blood sample.

48. A method for detecting gestational trophoblast disease in a sample from a subject comprising:

(a) contacting a sample with an antibody which specifically binds to the early pregnancy associated molecular isoform of hCG under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG;

(b) contacting the sample with a second antibody which specifically binds to intact non-nicked hCG without substantially cross-reacting with said antibody under conditions permitting formation of a complex between the antibody and the early pregnancy associated molecular isoform of hCG;

(c) measuring the amount of complexes formed, thereby determining the amount of the early pregnancy associated molecular isoform of hCG in the sample due to binding with the first antibody, and late pregnancy associated molecular isoform of hCG in the sample due to binding with the second antibody;

(d) determining the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the subject; and

(e) comparing the ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) over time, wherein a continuing high ratio of early pregnancy associated molecular isoform of hCG to late pregnancy associated molecular isoform of hCG in the sample determined in step (c) indicates the presence of gestational trophoblast disease in the subject.

49. The method of claim 48, wherein the antibody is B604, B151, B152 or B207.

50. The method of claim 48, wherein the second antibody is B108, B109.

51. The method of claim 48, wherein the gestational trophoblast disease is choriocarcinoma or hydatidiform mole.

52. The method of claim 48, wherein the sample is a urinary sample or a blood sample.

* * * * *

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L1   53494 S ASSAY AND IMMUNOASSAY
L2   58 S ANALYTE AND ISOFORM#
L3   9 S L1 AND L2
L4   8 DUP REM L3 (1 DUPLICATE REMOVED)

7/25/03